UNIVERSIDADE DE LISBOA
FACULDADE DE FARMÁCIA

Cyclodextrin supramolecular systems for oral antiretroviral drug delivery

Oluwatomide Adeoye

Orientador: Professora Doutora Helena Maria Cabral Marques
Co-orientadores: Professor Doutor Nuno Taveira

Tese especialmente elaborada para obtenção do grau de Doutor em Farmácia na especiladade de Tecnologia Farmacêutica

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2019

Esther Ọmóbóyèdé Àdùké

Òrìsà bí ìyá ò sí
Não há divindade no mundo como mãe

Stephen Olúwagbémiga Àrèmú

Bàbá ẹni níjó ogún le, bàbá ẹni níjó ìjì n jà
O pai na batalha, o pai na tempestade

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Abstract

The global pandemic of Human Immunodeficiency Virus (HIV) is responsible for the death of more than 39 million people. Currently, about 37 million people are infected with an estimated rate of 2.1 million new cases and 1.2 million HIV related deaths per annum. Over the past three decades, Antiretroviral Therapy (ART) has significantly improved HIV prognosis and health related quality of life. However, HIV still remains a significant public health and socio-economic concern due to the inability to cure or completely eradicate both long-lived latently infected cells and replication competent provirus from anatomical and lymphatic reservoirs. This has necessitated lifelong pharmacotherapy in order to keep the viral loads below detection and prevent progression of the infection to full blown Acquired Immune Deficiency Syndrome (AIDS). Presently, antiretroviral (ARVs) drugs used in the management of HIV are administered orally due to the volume and ambulatory nature of the lifelong therapeutic interventions. However, poor oral bioavailability, inadequate biodistribution and adverse side effects of many ARVs continue to preclude optimal therapeutic outcomes. In recent years, there has been an increased interest in the development of ARV delivery systems specifically tailored to improve HIV's therapeutic outcomes.

Within this context, cyclodextrins (CyD) based drug delivery systems offer several possibilities for constructing an ARV drug delivery system with enhanced bioavailability, biodistribution and therapeutic performance. The traditional role of cyclodextrins (CyDs) in the pharmaceutical industry has been to enhance the aqueous solubility, physicochemical and physiological stability, the deliverability and therapeutic performance, and reduce the adverse side effects of several active pharmaceutical ingredients (APIs). Also, CyDs have been reported to modulate cytochrome P450 (CYP450) metabolism and p-glycoprotein (P-gp) efflux transport for enhanced drug pharmacokinetic profiles.

Thus, this study was designed to explore the utility of CyD supramolecular systems in the development of an oral ARV delivery system with enhanced pharmacokinetic properties. Lopinavir (LPV), a potent inhibitor of HIV-1 protease and a Class 4 Biopharmaceutical Classification System (BSC) drug with poor oral bioavailability was chosen as our model drug. LPV's poor bioavailability is a combined effect of low aqueous solubility, P-gp efflux transport/low gastric permeability and CYP450 3A metabolism. Presently, LPV is clinically available as a co-formulation with suboptimal doses of ritonavir (RTV) which enhances oral bioavailability by inhibiting LPV's metabolism. The adverse side effects associated with RTV

has made necessary the development of a RTV free LPV formulation. Also, paediatric formulations of LPV (even with RTV) is fraught with problems such as the presence of high quantities of ethanol and propylene glycol in the commercially available oral solution.

Therefore, an *in silico* method was developed an validated experimentally (using ibuprofen as model drug) to study the host-guest molecular interaction between LPV and CyD, and to predict the best CyD molecule for developing a CyD based LPV delivery system. The predicted derivative of (2-hydroxyl)propyl-gamma-cyclodextrin (HP-γ-CyD) with a high degree of substitution (DS) that is, HP17-γ-CyD, was synthesized and evaluated comparatively with parent γ-CyD and commercially available HP-γ-CyD. The applicability of supercritical assisted spray drying (SASD), a non-toxic and cheap method for enhancing CyD complex formation was also evaluated. Then, polymer CyD (pCyD) sub-microcarriers were synthesized, loaded with LPV and evaluated for its ability to modulate drug release and enhance antiviral activity.

The results suggests the application of *in silico* methodologies is a feasible approach for the rational and/or deductive development of CyD based drug delivery systems. The predicted HP17-γ-CyD facilitated a higher LPV amorphization and solubilization relative to the parent γ-CyD and commercially available HP-γ-CyD. Also, the application of SASD technique for preparing LPV-CyD complexes enhanced drug solubilization and complexation. With regards to the sub-microcarrier, results indicate the successful synthesis of a water soluble p(CyD) based on (2-hydroxyl)propyl-beta-cyclodextrin (HP-β-CyD) and methyl-beta-cyclodextrin (M-β-CyD) as monomers, and pyromellitic dianhydride as crosslinker. The physicochemical analysis of the LPV loaded pCyD revealed the successful preparation of sub-micron sized particles with good encapsulation efficiencies, a pCyD mediated amorphization of LPV and a significant increase in LPV solubilization and release. *In vitro* cytotoxicity of the pCyD and their LPV formulations revealed good safety profiles in Caco-2 and Sup-T1 cell lines. Results obtained from the *in vitro* antiviral assays revealed a dose independent HIV-1 inhibition by the synthesized pCyD and a CyD monomer dependent synergistic antiviral activity in their formulations.

Overall, the study contributed to a better understanding of *in silico* methodologies as predictive tools for CyD selection in drug development. It was possible to completely avoid the tedious and time/resource consuming traditional approach of selecting CyD by calculating association constants from phase solubility studies or isothermal titration calorimetry. The

application of SASD for drug-CyD complexation seems an interesting alternative to conventional spray drying and other methods of CyD solid complex formation. Also, considering the potential for drug dose reduction without a compromise on antiretroviral activity, the synthesized pCyDs represent a promising approach for formulating new alternatives to clinically available antiretroviral drugs. In conclusion, all the results obtained in this experimental work showed the interest of CyDs as promising vehicles for the development of ARV drug delivery systems with enhanced pharmacokinetic profiles.

Keywords: Cyclodextrin, Lopinavir, Supercritical Assisted Spray Drying, Molecular Docking and Dynamics, HIV/AIDS, Complex Formation

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Resumo

A pandemia global do vírus da imunodeficiência humana (VIH) é responsável pela morte de mais de 39 milhões de pessoas. Atualmente, aproximadamente 37 milhões de doentes estão infetados apresentando uma taxa estimada de 2,1 milhões de novos casos e 1,2 milhões de mortes anuais. Nas últimas três décadas, fármacos antiretrovirais permitiram um aumento no diagnóstico e qualidade de vida associada aos portadores do referido vírus. Contudo, a infeção pelo vírus da imunodeficiência humana continua uma temática do quotidiano, tanto a nível da saúde pública, como dos aspectos sócio-económicos devido à incapacidade de curar ou erradicar totalmente as células infetadas e a replicação do provírus por parte dos reservatórios anatómicos e linfáticós. Os aspetos referidos tornam necessários a administração a longo prazo de terapias de forma a preservar a carga viral abaixo da sua deteção e a prevenção da progressão da infeção até ao síndrome de deficiencia humana adquirida (SIDA). Presentemente, os fármacos antiretovirais (ARVs) são administrados por via oral devido à natureza ambulatória e ao volume das intervenções atualmente empregues na terapêutica de longa duração. Não obstante, a baixa e inadequada biodistribuição de vários ARVs continua a dificultar um adequado tratamento. Nos últimos anos, tem sido notório o um crescente aumento no desenvolvimento de sistemas de veiculação contendo ARV especificamente desenhados para uma melhor terapêutica para o VIH.

Neste contexto, os sistemas de libertação de fármacos baseados na utilização de ciclodextrinas (CyDs) oferecem uma metodologia competente na definição de um sistema de libertação de fármacos antiretrovirais e que permitiram vantagens na biodisponibilidade, biodistribuição e atividade terapêutica dos mesmos. A indústria farmacêutica tem, nos últimos anos, utilizado ciclodextrinas como uma forma de: aumentar a solubilidade e dissolução em água de muitos fármacos, assim como aumentar a estabilidade físico-química e fisiológica e a distribuição e a atividade dos mesmos. A utilização de ciclodextrinas também tem sido referida como uma metodologia que permite a modulação do metabolismo do citocromo P450 (CYP450) e do efluxo da p-glicoproteína (P-gp) melhorando o perfil farmacocinético dos fármacos.

Desta forma, o presente estudo foi desenvolvido com o objetivo de avaliar a aplicabilidade de sistemas supramoleculares contendo CyDs no desenvolvimento de sistemas de veiculação orais contendo antiretrovirais com propriedades farmacocinéticas relevantes. Lopinavir

(LPV), um inibidor da protease VIH-1 pertencente à classe 4 do sistema de classificação biofarmacêutica foi utilizado no presente estudo como um fármaco modelo. A baixa biodisponibilidade apresentada pelo LPV resulta de um efeito cumulativo da sua baixa solubilidade em água, da baixa permeabilidade gástrica/transporte de efluxo da P-gp e do metabolismo do CYP450 3A. Atualmente, a administração de LPV está disponível como uma co-formulação suplantada com doses de ritonavir (RTV), as quais permitem um aumento da biodisponibilidade oral através da inibição do metabolismo do LPV. Contudo, os efeitos adversos apresentados pelo RTV tornaram necessário o desenvolvimento de uma formulação de LPV sem RTV na sua constituição. Mais ainda, formulações pediátricas de LPV (mesmo apresentando RTV) apresentam inúmeros problemas tais como a presença de elevadas quantidades de etanol e propilenoglicol nas soluções orais existentes atualmente.

Assim, um método *in silico* foi desenvolvido e validado experimentalmente (através da utilização de ibuprofeno como fármaco modelo) de modo a estudar a interação molecular entre o LPV e diferente tipos de CyDs e de forma a prever qual a melhor CyD para desenvolver um sistema libertação de LPV baseado em CyD. O derivado previsto de (2-hidroxil) propil-gama-ciclodextrina (HP-γ-CyD) com um elevado grau de substituição, isto é, HP17-γ-CyD, foi sintetizado e comparado com a γ-CyD original e HP-γ-CyD derivada comercialmente disponíveis. Foi ainda avaliada a aplicabilidade da secagem por atomização assistida por fluidos supercríticos (SASD), um método não tóxico e barato para otimizar a produção de complexos de CyDs. Em seguida, as partículas com o polímero CyD (pCyD) foram sintetizadas, encapsuladas com o LPV e avaliado quanto à sua capacidade de modular a libertação de fármaco e aumentar a atividade antiviral.

Os resultados apresentados permitiram concluir que a aplicação de metodologias *in silico* é uma abordagem exequível para o desenvolvimento racional e/ou dedutivo de sistemas de administração de medicamentos baseados em CyDs. O complexo previsto HP17-γ-CyD facilitou uma maior amorfização e solubilização do LPV em relação às γ-CyD e HP-γ-CyD comercialmente disponíveis. Além disso, a aplicação da técnica SASD para a preparação de complexos LPV-CyD permitiu a maior solubilização e complexação do fármaco. Em relação ás partículas, os resultados indicam a síntese de um pCyD solúvel em água com base em monómeros de (2-hidroxil)propil-beta-ciclodextrina (HP-β-CyD) e metil-beta-ciclodextrina (M-β-CyD) e dianidreto piromelítico como cross-linker. A análise físico-química do pCyD carregado com LPV revelou a preparação, com sucesso, de partículas de dimensão submícrométrica com boas eficiências de encapsulação, uma amorfização do LPV mediada

por pCyD e um aumento significativo na solubilização e libertação do LPV. A citotoxicidade *in vitro* das formulações de pCyD e LPV revelou bons perfis de segurança nas linhas celulares Caco-2 e Sup-T1. Os resultados obtidos a partir dos ensaios antivirais in vitro revelaram uma inibição independente da dose do HIV-1 pela pCyD sintetizada e uma atividade antiviral sinérgica dependente do tipo de CyD nas suas formulações.

No geral, o estudo contribuiu para uma melhor compreensão das metodologias *in silico* como ferramentas preditivas para a seleção de CyD no desenvolvimento de fármacos. Foi também possível evitar completamente a abordagem tradicional tediosa e demorada relativamente à seleção da CyD através do cálculo de constantes de associação a partir de estudos de solubilidade de fase ou da calorimetria de titulação isotérmica. A aplicação do SASD para a complexação de fármacos-CyDs parece ser uma alternativa viável à secagem por aspersão convencional e outros métodos de formação de complexos sólidos de CyDs. Além disso, considerando o potencial de redução da dose do medicamento sem comprometer a atividade anti-retroviral, os pCyDs sintetizados representam uma abordagem promissora para a formulação de novas alternativas aos fármacos ARV disponíveis clinicamente. Em conclusão, todos os resultados obtidos neste trabalho experimental mostraram o interesse das CyDs como veículos promissores para o desenvolvimento de sistemas de administração de fármacos ARV com melhores perfis farmacocinéticos.

Palavras Chave: Ciclodextrinas, Lopinavir, Atomização Assistida por Fluídos Supercríticos, VIH/SIDA, Formacão de Complexo

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List of Abbreviations and Acronyms

^{13}C CP/MAS NMR	^{13}C cross-polarization magic angle spinning nuclear magnetic resonance
^{1}H-NMR	Proton nuclear magnetic resonance
3TC	Lamivudine
ABC	Abacavir
AIDS	Acquired immune deficiency syndrome
APIs	Active pharmaceutical ingredients
ART	Antiretroviral therapy
ARV	Antiretroviral drugs
ATB	Automated Topology Builder
ATR-FTIR	Attenuated total reflectance Fourier transform infrared spectroscopy
AZT	Zivovudine
BCS	Biopharmaceutical classification system
Caco-2	Human colon carcinoma cell line
CE	Complexation efficinecy
CGTase	Cyclodextrin glucosyltransferase enzyme
CoEva	Co-evaporation
CyD	Cyclodextrin
CyD-NS	Cyclodextrin nanosponges
CYP P450	Cytochrome P450
DMF	Dimethylformamide
DM-β-CyD	Dimethyl-beta-cyclodextrins
DRV	Darunavir
DS	Degree of substitution
DSC	Differential scanning calorimetry
DTG	Dolutegravir
EFV	Efavirenz
ESR	Electron spin resonance
Et_3N	Triethylamine
FTC	Emtricitabine
FTIR	Fourier-transform infra-red
GALT	Gut associated lymphoid tissues
GAS	Gaseous antisolvent
GIT	Gastrointestinal tract
GRAS	Generally recognized as safe
HIV	Human immunodeficiency virus
HP16-γ-CyD	(2-hydroxy)propyl-gamma-cyclodextrin (DS=16)
HP17-γ-CyD	(2-hydroxy)propyl-gamma-cyclodextrin (DS≈17)
HP-β-CyD	(2-hydroxy)propyl-beta-cyclodextrin
HP-γ-CyD	(2-hydroxy)propyl-gamma-cyclodextrin

HSM	Hot stage microscopy
HSQC-DEPT	Heteronuclear single quantum coherence spectroscopy-distortionless enhancement by polarization transfer
HSQC-NMR	Heteronuclear single quantum coherence spectroscopy nuclear magnetic resonance
IBU	Ibuprofen
ISTIs	Integrase strand transfer inhibitor
ITC	Isothermal titration calorimetry
$K_{1:1}$	Stability constant
LPV	Lopinavir
MD	Molecular dynamics
MIP	Molecular imprinted polymers
NMR	Nuclear magnetic resonance
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside reverse transcriptase inhibitors
NVP	Nevirapine
PCA	Particles by compressed antisolvent
pCyD	Polymer(ic) cyclodextrin
P-gp	P-glycoproteins
pHPβCyD	Polymer-hydroxypropyl-beta-cyclodextrin
PIs	Protease inhibitors
PM	Physical mixture
PMDA	Pyromellitic dianhydride
pMβCyD	Polymer-methylated-beta-cyclodextrins
PVA	Polyvinyl alcohol
PVP	Polyvinylpyrrolidone
RESOLV	Rapid Expansion of a Supercritical Solution into a Liquid Solvent
RESS	Rapid Expansion of Supercritical Solution
RM-β-CyD	Ramdomly-methylated-beta-cyclodextrins
RTV	Ritonavir
SAS	Supercritical antisolvent
SASD	Supercritical CO_2 assisted spray drying
SBE-β-CyD	Sulfobutylether-beta-cyclodextrins
SCFs	Supercritical fluids
SEDS	Solution enhanced dispersion supercritical fluids
SEM	Scanning electron microscopy
Sup-T1	T cell lymphoblastic lymphoma cell line
$TCID_{50}$	The 50% tissue culture infectious dose
TDF	Tenofovir
TGA	Thermo gravimetric analysis
TZM-bl	Recombinant HeLa cell line
UV	Ultraviolet/visibble spectroscopy
XRD	X-ray diffraction

α-CyD	Alpha-cyclodextrin
β-CyD	Beta-cyclodextrin
γ-CyD	Gamma-cyclodextrin
δ-CyD	Delta-cyclodextrin
ΔE	Complexation energy
ε-CyD	Epsilon-cyclodextrin
ζ-CyD	Zeta-cyclodextrin
η-CyD	Eta-cyclodextrin

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Aims and Organization of the Thesis

One of the key issues associated with lifelong HIV pharmacotherapy is the increased incidence of severe acute and/or chronic adverse drug effects. These adverse effects are often mediated by poor drug tolerability, drug-drug interactions and toxicities that can exacerbate poor patient adherence and lead to increased incidence of resistant strains of the virus. One of the main approaches being used to address these issues is the development of new antiretroviral (ARV) drug delivery systems with enhanced pharmacokinetic, pharmacodynamic and safety (reduced side effects) profiles.

The main objective of the scientific work leading to this doctoral thesis was to investigate the utility of cyclodextrin (CyD) supramolecular systems in the development of an oral ARV delivery system with enhanced pharmacokinetic properties. Lopinavir (LPV), currently recommended by the World Health Organisation as part of the HAART regimen was selected as a good model for some of the key issues associated with oral antiretroviral drugs (ARVs): (i) it has a low oral bioavailability due to a combined effect of low aqueous solubility and P-glycoprotein (P-gp)/CYP450 3A mediated low gastric permeability and metabolism; (ii) it is clinically co-formulated with suboptimal doses of ritonavir (RTV) which enhances oral bioavailability by inhibiting LPV's metabolism, but also contributes significantly to adverse drug effects; and (iii) paediatric oral solutions of LPV are currently formulated with 42%$^{w}/_{v}$ of alcohol and 15.3%$^{w}/_{v}$ propylene glycol; which is clearly undesirable. In order to investigate CyDs' utility in resolving these shortcomings of LPV, three specific aims were defined for the study:

i. to study the molecular interaction between LPV and several CyD molecules in order to select the best CyD molecule for complex formation;
ii. to explore and integrate a downstream pharmaceutically scalable drug formulation processing technology for the controlled preparation of CyD complexes and to evaluate its utility relative to other methods, and;
iii. to develop a polymer CyD based LPV sub-microcarrier and evaluate its ability to enhance drug bioavailability and antiviral activity.

All the experimental studies described in this thesis, which began in April 2015, were performed at the Departamentos de Farmácia Galénica e Tecnologia Farmacêutica, Químíca Farmacêutica e Terapêutica, and Microbiologia e Imunologia of the Faculty of Pharmacy,

Universidade de Lisboa; Departamento de Químíca, Faculty of Science and Technology, Universidade Nova de Lisboa and at the Department of Drug Science and Technology, University of Turin, Italy.

This thesis is divided into 5 chapters. Each chapter is presented in the form of a research article that has either been published or is being prepared for submission. The first chapter is a general introduction to describe some of core thematic topics addressed in the thesis while the following 3 chapters describes the experimental work performed. The last chapter presents the concluding remarks and future work arising from the thesis. The following paragraphs summarize the content of each chapter

Chapter 1 consists of the general introduction and is divided into three sections as described below:

- In **Section 1,** CyDs' structure, physicochemical properties and mechanism supporting its pharmaceutical applications are discussed.
- **Section 2** provides a general overview of pharmaceutical nanotechnology in oral delivery systems and the intelligent design of CyD nanosystems to achieve optimal bioavailability and biodistribution.
- In **Section 3,** HIV/AIDS is reviewed focussing on oral pharmacotherapy and the current/emerging drug delivery strategies being developed to address issues of bioavailability, biodistribution, adverse drug effects and medication compliance.

Chapter 2 is divided into two sections, which describes the preliminary studies on the host-guest interaction between several CyD molecules and LPV, and the utility of supercritical fluid assisted spray drying in the preparation of CyD complexes.

- In **Section 1,** an *in silico* method was developed to screen and select the best CyD for LPV complexation and solubilization. The *in silico* method using Molecular docking protocol on the Molecular Operating Environment software was first applied to ibuprofen, a non-steroidal anti-inflammatory drug whose complexation behaviour with CyD is fully understood. The results were validated experimentally by correlating the rank order of the *in silico* derived complexation energy with that of the experimentally derived association constants (phase solubility studies). The validated

in silico method was then used to screen LPV-CyD host guest interactions in order to select the best CyD.

- **Section 2** describes the application of Supercritical CO_2 assisted spray drying for the preparation of drug-CyD complexes. Again, ibuprofen was used as a model drug and the prepared complexes were fully characterized by ATR-FTIR, XRD, UV, ^{13}C CP/MAS NMR, SEM and DSC

Chapter 3 comes in the sequence of the previous chapter. Based on the *in silico* results in Chapter 2, a CyD derivative i.e. (2-hydroxyl)propyl-gamma-Cyclodextrin (HP-γ-CyD) with a high degree of substitution (DS, 16) was identified and subjected to further *in silico* studies (molecular dynamics) in order to gain more insights into the nature of the molecular interaction with LPV. This selected CyD derivative was then synthesized and evaluated comparatively with parent γ-CyD and commercially available HP-γ-CyD (DS, 4.6). The complexes were prepared by supercritical fluid assisted spray drying (SASD) and co-solvent evaporation (CoEva) were fully characterized by ATR-FTIR, XRD, ^{13}C CP/MAS NMR, and *in vitro* dissolution studies.

Chapter 4 reports the synthesis, characterization and development of a polymeric CyD (pCyD) sub-microcarrier based on pyromellitic dianhydride (PMDA) and either methyl-beta-cyclodextrin (Me-β-CyD) or (2-hydroxyl)propyl-beta-cyclodextrin HP-β-CyD. Colloidal complexes of LPV with the pCyD were prepared and the formulations fully characterized for morphology, physicochemical properties, drug release, and *in vitro* cytotoxicity and antiviral activity.

Chapter 5 summarizes the main conclusions of the experimental work, highlights the limitations and discusses the future perspectives/work arising from this study.

CHAPTER 1

General Introduction

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Section 1

Cyclodextrins: Structure, physicochemical properties and pharmaceutical applications

This section was partially adapted from the book chapter:

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1. Introduction

Cyclodextrins (CyDs) are cyclic oligosaccharides made up of repeating glucopyranose units linked in a ring formation by α-(1,4) glycosidic bonds (Figure 1.1.1). They were first isolated and reported in 1891 by the French scientist Villiers, as the product of bacteria (Bacillus amylobacter) induced degradation of starch. The study of CyDs chemistry in the early twentieth century led to the identification of the first generation CyDs, i.e. alpha (α-CyD), beta (β-CyD), and gamma-cyclodextrin (γ-CyD), and the subsequent biosynthesis of pure CyD [1-8].

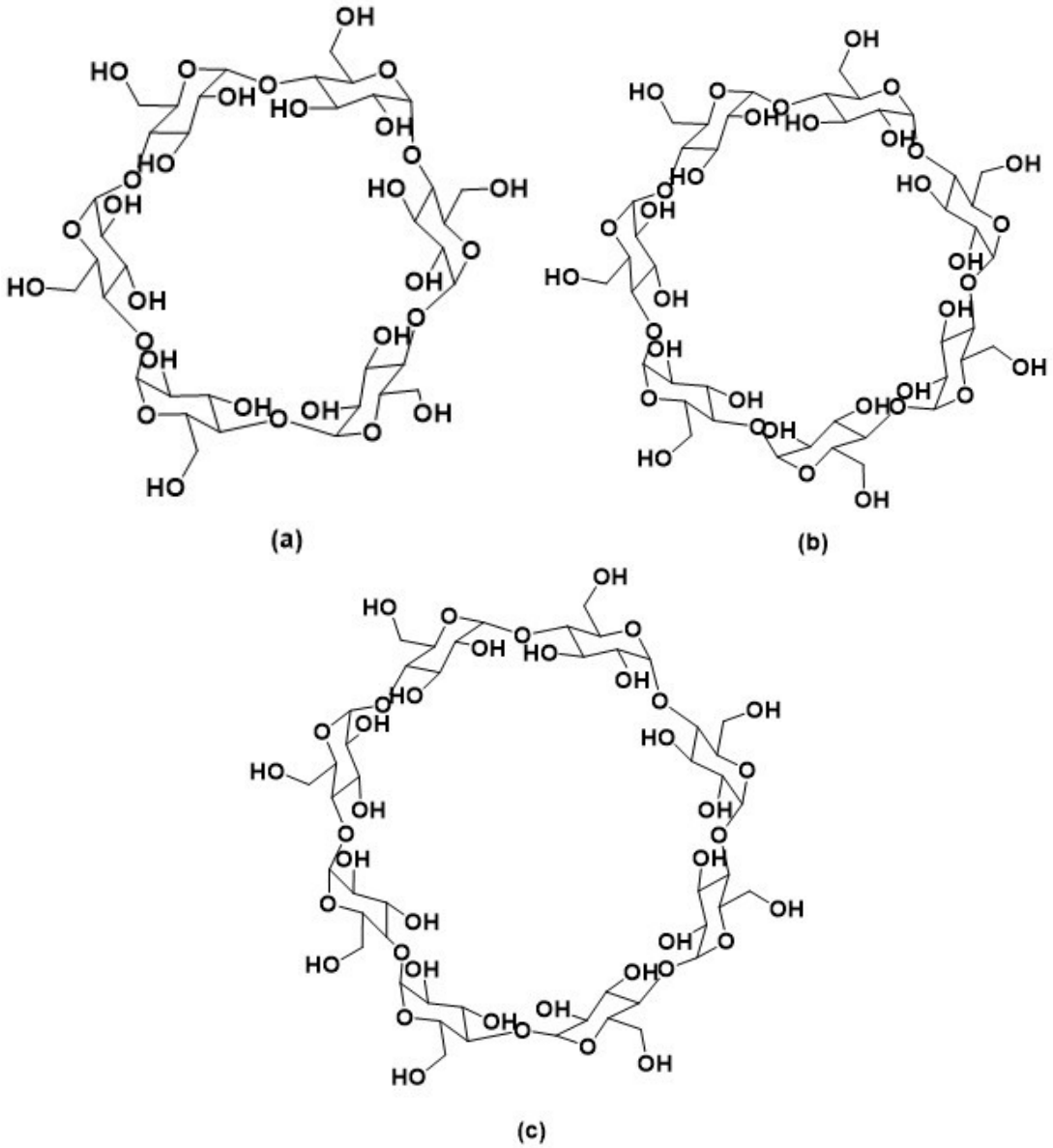

Figure 1.1.1: Schematic representation of the chemical structure of (a) α-CyD, (b) β-CyD and (c) γ-CyD

The biosynthesis of CyD is facilitated by an enzymatic product of bacteria known as cyclodextrin glucosyltransferase enzyme (CGTase). The CGTase enzyme can be produced from different types of microorganisms such as strains of Bacillus sp. (B. amylobacter, B. circulans, B. megaterium, B. subtilis, B. macerans, alkalophilic Bacillus sp.), Klebsiella (K. pneumoniae, K. oxytoca), Thermoanaerobacter sp., Clostridium sp.,[8]. During the process, CGTase induces the intra-molecular trans-glycosylation reaction to degrade the amylase helix fraction of starch, and facilitate the subsequent cyclisation of the product to form toroidal or doughnut shaped molecules (Figure 1.1.2). This toroidal shape is due to the stabilization of the C2-C3 hydroxyl groups by hydrogen bonds, and its rigid non-rotating structure. The most important characteristic of the CyD toroids is the hydrophilic exterior surfaces and hydrophobic interior cavities which confers on CyD, the ability to clathrate various molecules, thus forming inclusion complexes. This toroidal characteristic is due to the orientation of the hydroxyl groups of the glucose residues towards the external surfaces of the toroid with the primary (C-6) and secondary (C-2 and C-3) hydroxyl groups at the narrow and wider edges, respectively. On the other hand, the interior cavity is lined by skeletal carbons and ethereal oxygens which give it a lipophilic character [6,9-13]. Many types of starch have been used as substrates for CyD biosynthesis. However, potato starch is still the most commonly used due to high yield [13].

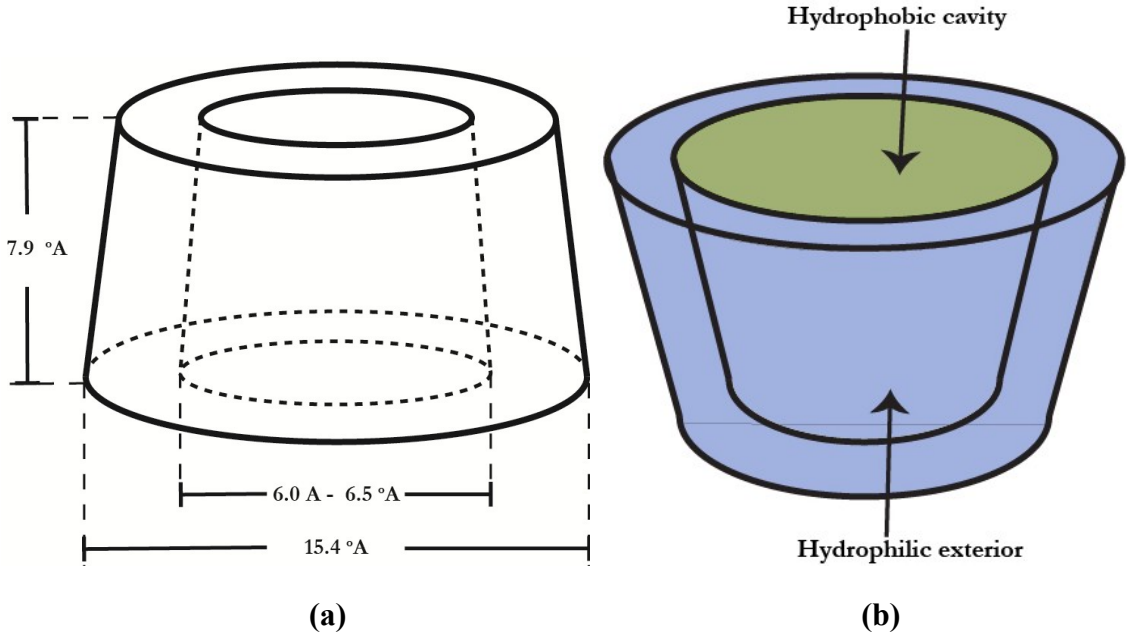

Figure 1.1. 2 : Toroidal structure of β-CyD.

The natural CyDs i.e. α-CyD, β-CyD and γ-CyD are formed by six, seven and eight glucopyranose units respectively [7]. While CyD with fewer glucose units cannot be formed due to steric hindrances, homologs with nine or more glucose units have been identified. French and co-workers [14] identified delta (δ-CyD) and epsilon (ε-CyD) with nine and ten glucopyranose units respectively. Thoma and Stewart [15] identified zeta (ζ-CyD) and eta (η-CyD) with eleven and twelve glucopyranose units respectively. Unlike the active CyDs, these larger CyDs have small biosynthetic yields, are difficult to purify and also have poor molecular inclusion properties [8,16,17]. Natural CyDs are crystalline, homogeneous, and non-hygroscopic substances. Their melting point is not clearly defined as they start to decompose at about 270 ^{o}C and produce a sharp endothermic endpoint (differential scanning calorimetry) indicating CyD decomposition around 300 ^{o}C [8]. Some other important physicochemical properties [18,19] of natural CyD are presented in Table 1.1.1

2. Cyclodextrin Derivatives

The developments of biotechnological and industrial chemistry processes of 20th century led to the synthetic or semi-synthetic modification of many natural molecules. These modification processes were carried out to enhance desired physicochemical properties while reducing the unwanted ones. These technologies also led to the synthesis of many CyD derivatives by molecular substitution (amination, esterifications, etherifications) of their primary and secondary hydroxyl groups. Depending on the nature and degree of substitution, the hydrophobic molecular cavity volume, affinity for guest molecules, solubility, and stability of natural CyD were modified [20-23].

For instance, while natural CyD are hydrophilic, their aqueous solubility is limited. This aqueous solubility which is dependent on hydrogen bonding between hydroxyl groups is dramatically increased by substitution of the hydroxyl groups. Even substitution by hydrophobic moieties such as methoxy functions can increase aqueous solubility [24,25]. The use of hydropropyl derivative of β-CyD (HP-β-CyD) while increasing aqueous solubility also reduces the drug binding ability of the CyD cavity. However, the drug binding ability of these parent CyD (β-CyD) can be enhanced by partial methylation of the hydroxyl groups 2-, 3- and 6- positions at the risk of increasing its toxicity [26]. Another group of derivatives are the amphiphilic CyDs, formed by primary and/or secondary side chain substitution with long alkyl and fluoroalkyl groups. They are important in dermal drug delivery due to their ability

to form monolayers, micelles and bilayer vesicles [28]. While more than a hundred derivatives of CyDs have been synthesized, toxicological and safety concerns have limited the approval and application of many as drug carriers for therapeutics [29]. Some common CyD derivatives are listed in Table1.1.2.

Table1.1.1: Physicochemical properties of natural cyclodextrins [adapted from [27]]

Properties	α	β	γ
No. of glucose units	6	7	8
Molecular weight	972	1135	1297
solubility in water (g/100 ml), room *T*	14.5	1.85	23.2
$[R]_D$ 25 ^oC	150.0 ±0.5	162.5 ±0.5	177.4±0.5
Inner cavity diameter (Å)	4.7 -5.3	6.0 -6.5	7.5-8.3
Height of Torus (Å)	7.9 ±0.5	7.9 ±0.5	7.9 ±0.5
Diameter of outer periphery (Å)	14.6 ±0.4	15.4 ±0.4	17.5 ±0.4
Aproximate cavity volume ($å^3$)	174	262	427
Aproximate cavity volume in 1 mol CyD(ml)	104	157	256
Aproximate cavity volume in 1g CyD(ml)	0.1	0.14	0.2
Crystalline forms (from water)	hexagonal plates	Monoclinic parallelograms	Quadratic prisms
Crystal water content (% wt)	10.2	13.2-14.5	8.13-17.7
Water molecules in cavity	6	11	17
Diffusion constant at 40 ^oC	3.443	3.224	3
Hydrolysis by *A. oryzae* α-amylose	Negligible	Slow	Rapid
pK (by potentiometry) at 25 ^oC	12.332	12.202	12.081
Surface tension (MN/m)	71	71	71
Partial molar volumes in solution (ml/mol)	611.4	703.8	801.2
Adiabatic compressibility in aqueous solutions ml (mol^{-1} bar^{-1})x 10^4	7.2	0.4	-5

Table 1.1.2: Physicochemical properties of natural cyclodextrins and their derivatives [adapted from [7]]

Cyclodextrin Derivatives		Molecular Weight	Solubility in Water (mg/ml at 25°C)
Alpha-Cyclodextrin	α-CyD	972	145
Beta-Cyclodextrin	β-CyD	1135	18.5
2-Hydroxylpropyl-Beta-Cyclodextrin	HP-β-CyD	1400	> 600
Dimethyl-Beta-Cyclodextrin	DM-β-CyD	1331	
Ramdomly-Methylated-Beta-Cyclodextrin	RM-β-CyD	1312	>500
Sulfobutylether-Beta-Cyclodextrins	SBE-β-CyD	2163	>500
Gamma-Cyclodextrin	γ-CyD	1297	232
2-Hydroxylpropyl-Gamma-Cyclodextrin	HP-γ-CyD	1576	>500

3. Cyclodextrin (host) - guest molecular interaction and pharmaceutical applications

The pharmaceutical applications of CyDs are mainly derived from their ability to form complexes (inclusion, non-inclusion complexes and aggregates) with guest hydrophobic molecules (drugs) in an aqueous medium [18,27,30]. Thus, CyDs are able to modify the physicochemical and pharmacokinetic properties of drug molecules such as drug solubility, bioavailability, membrane permeability, enzymatic and chemical stability, photostability, physical state, taste and odour, skin irritability, intrinsic volatility, etc; for efficient drug delivery [11]. Drug-CyD inclusion complexes are formed by non-covalent bonds or simply by the clathration of the guest molecule into the toroidal hydrophobic cavity of CyD (Figures 1.1.3 and 1.1.4). During inclusion complex formation, whole drug molecule or some lipophilic moiety of a compound with poor aqueous solubility replaces enthalpy rich water molecules thus reducing the energy of the system. The equilibrium between the guest molecule and free CyD facilitates inclusion complex formation. The efficiency of this inclusion process depends on the polarity, steric dimensions, molecule size, electronic effects, temperature, pH and method of inclusion. Other factors like the hydrogen bonding, van der Waals, surface tension, molar ratio (e.g. 1:1, 1:2, 2:1), have also been reported to play some role in the formation of inclusion complexes (Figure 1.1.4). It is important to note that not all drug molecules form stable inclusion complexes.

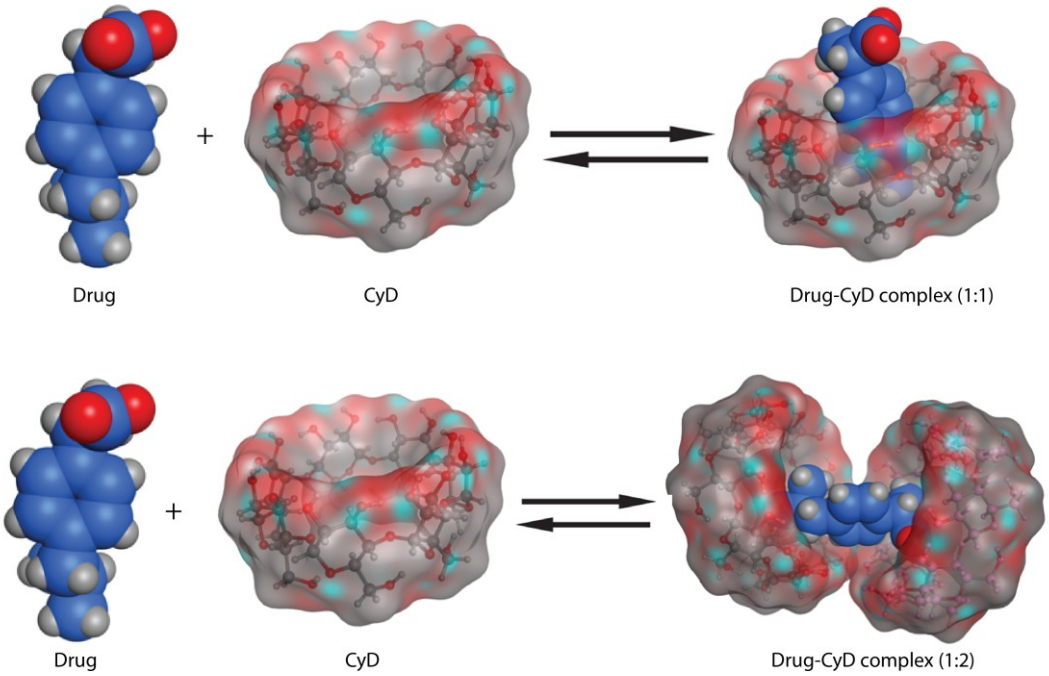

Figure 1.1. 3 : Drug–CyD complex (1:1) and (1:2) stoichiometric ratio.

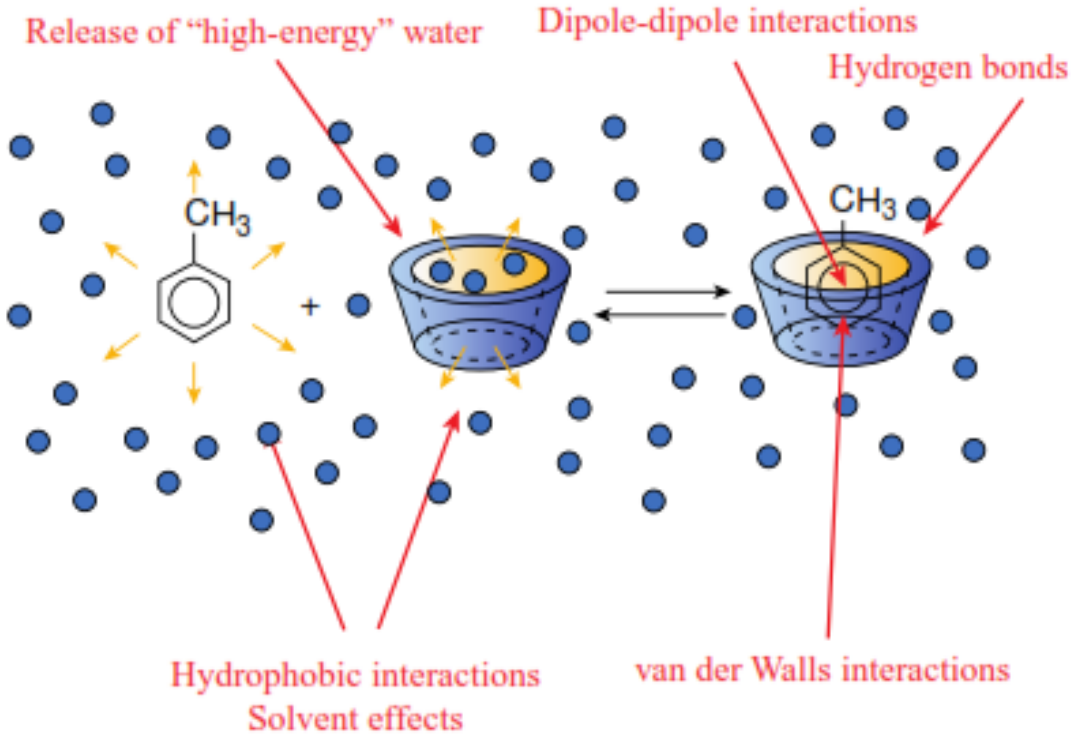

Figure 1.1. 4: Driving force for complex formation [8]

Molecules with high aqueous solubility tend to interact with the hydrophilic surfaces to form association compounds or non-inclusion complexes [9,12]. Several analytical techniques are used to detect the formation of CyD complexes in both aqueous solution and solid states. Examples include; spectroscopic techniques (Ultraviolet/visible (UV), Circular dichroism, Nuclear magnetic resonance (NMR), Electron spin resonance (ESR), Fluorescence, Fourier-transform infra-red (FTIR) and Raman), Polarimetry (isotermal titration calorimetry (ITC)), thermal analysis techniques (Differential scanning calorimetry (DSC), Thermal gravimetric analysis (TGA), Hot stage microscopy (HSM) etc. [31,32].

In oral drug delivery, CyDs affect the kinetics of key processes influencing gastric dissolution, absorption and permeability of dosage forms [27,33]. Thus, CyDs have been specifically used to increase the aqueous solubility of hydrophobic drugs, protect the labile drug molecules from corrosive environmental effects of the gastrointestinal tract, and also from potential degradation due to hydrolysis, oxidation, racemisation, enzymatic decomposition etc [24,34]. Also, CyDs have been used to modify the release profile of drugs for enhanced or controlled pharmacokinetic profiles [27]. Prolonged drug action, improved bioavailability and reduced side effects have been reported after oral administration of CyD complexes (alone or in ternary systems) of various hydrophobic drugs [35-37]. CyDs have also been used as controlled-release carriers for hydrophilic drugs [38-41]. Many CyD based oral drug delivery systems such as; floating and gastroretentive effervescent/non-effervescent tablets [42,43], mucoadhesive [44-46] , osmotic pump [47-49] and colon specific [50-52] have been developed.

4. Cyclodextrin safety/toxicity considerations in oral drug delivery

As with all other excipients used in the development of drug delivery systems, safety and toxicological considerations remains a major concern when seeking regulatory approval. The biological fate and toxicological profile of CyDs or other types of excipients are required to be fully defined. In order to address this concerns, the pharmacokinetics of several CyD molecules has been studied in humans and they are "Generally Recognized as Safe (GRAS)" [53-56]. For orally administered formulations, CyD's non-toxicity is primarily due to its non absorption from the gastrointestinal tract. Bioavailability is typically below 4 % with the exception of RM-β-CyD, whose higher values (≈1 %) are due to its surface activity [17,57].

While γ-CyD is completely digested in the gastrointestinal tract, α-CyD, β-CyD and CyD derivatives have been reported undergo bacteria induced digestion in the colon [58].

5. Conclusions

While CyD have been available for over a century, their application in the pharmaceutical industry is just about four decades old. Presently, there are about many CyD containing pharmaceutical products approved for use. More recently, a considerable amount of research effort have been concentrated on the development of CyD nanosystems in an attempt to combine the known benefits of CyD and nanotechnology based drug delivery. The theoretical considerations and application of these type of systems in oral drug delivery system is the subject of Section 2 of this chapter.

6. References

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Section 2

Cyclodextrins nanosystems in oral drug delivery: A mini review

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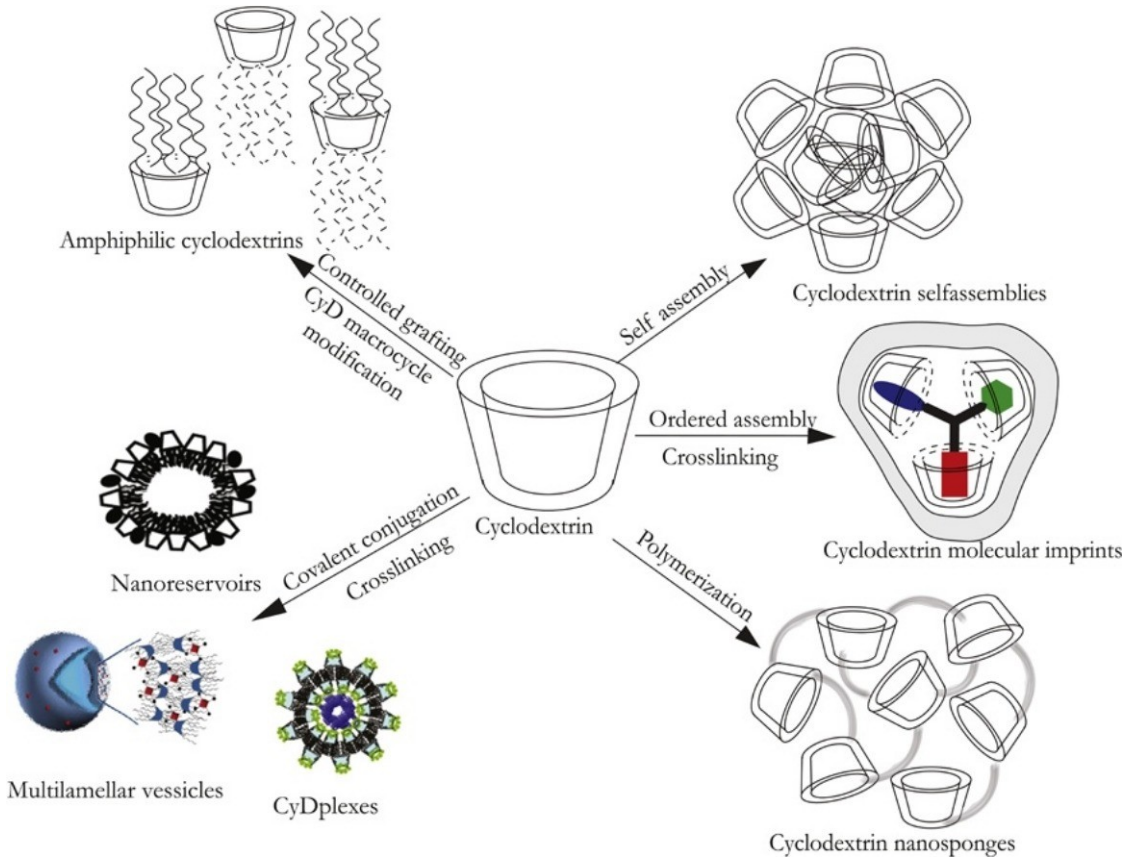
Abstract

Oral delivery of many therapeutic agents remains challenging due to gastric insolubility/poor dissolution, inefficient intestinal permeability and pre-systemic inactivation. These problems limit the advantages of convenience and increased compliance they provide in the therapy of many chronic diseases. Cyclodextrin nanosystems have emerged as promising platforms for drug-specific construction of the oral delivery nanosystems able to optimize the desired physicochemical properties and pharmacokinetic parameters; without a compromise on safety. This review focuses on some recent and encouraging advances in the application of cyclodextrin nanosystems for oral drug delivery. A general overview of cyclodextrins and pharmaceutical nanotechnology in oral delivery systems is provided. Some of the strategies being exploited for the synthesis of these nanosystems, and their potential for the intelligent navigation of the gastrointestinal tract for optimal bioavailability and biodistribution are then illustrated. Perspectives for translating these nanosystems from laboratory efficient formulations to clinically useful medicines are also discussed.

Graphical Abstract



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1. Introduction

The traditional role of cyclodextrins (CyDs) in the pharmaceutical industry has been to enhance the aqueous solubility, physicochemical and physiological stability, and the deliverability of various active pharmaceutical ingredients (APIs) [1-3]. Because of their structural arrangement (Figure 1.2.1), CyDs clathrate and form inclusion complexes with various APIs through non-covalent, host-guest interactions; thus modifying their physicochemical properties and *in vivo* behaviour [4]. Other forms of complexes such as the non-inclusion or association complexes have also been described in literature, and the nature of these interactions influences CyD's utility as drug delivery carriers [5].

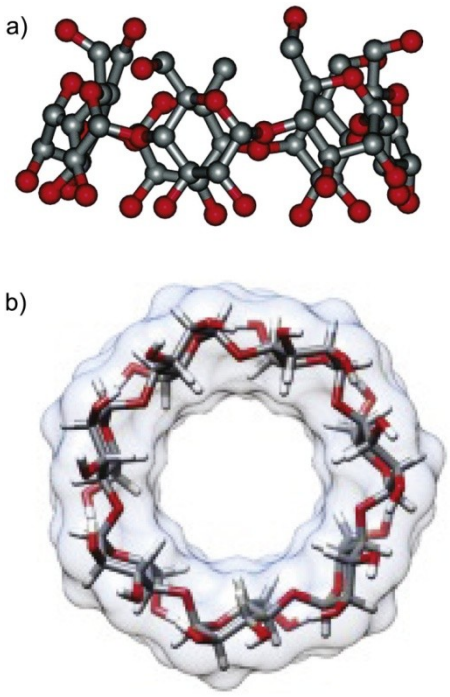

Figure 1.2.1: (a) Molecular structure of β-cyclodextrin. The red and gray tones are used to represent oxygen and carbon atoms, respectively while hydrogen atoms are omitted for clarity [6] (b)Toroidal, hollow, truncated cone structure of β-CyD [7].

From an oral drug delivery standpoint, CyDs have increased the concentration of APIs reaching systemic circulation or desired target site, from the gastrointestinal systems (GIT). This has led to a net positive effect on the pharmacologic activity and eventual treatment outcomes [8,9] . However, advances in pharmaceutical nanotechnology over the past two decades have further created vast opportunities for using complex CyD nanosystems to effectively modify and deliver small molecular drugs and biopharmaceuticals. The principal

advantage of CyD nanosystems is the provision of hybrid functionalities required to efficiently manipulate physicochemical and complex biological mechanisms that influence optimal drug bioavailability and biodistribution [10]. This multifunctionality is particularly desirable in oral therapeutics as it enables the provision of a single solution to a myriad of drug delivery challenges. Typically, an ideal oral nanosystem is expected to provide high drug loading, optimize gastric solubility and/or intestinal permeability, ensure controlled/targeted drug release, modulate a reduction in side effects, and protect the API from pre-systemic inactivation. While this holy grail of oral drug delivery is quite difficult to achieve, the versatility of a polymer like CyD allows for drug-specific construction of the delivery nanosystem able to optimize the desired physicochemical properties and pharmacokinetic parameters [11].

Cyclodextrins' multifunctional use in nanomedicine is underlined by four important properties. Firstly, CyD molecules are able to protect and shield APIs from specific and non-specific interactions in the physiological media [12,13] . Secondly, they can interact with and destabilize biological membranes to enhance drug permeability [13]. Thirdly, they have the ability to modulate the rate and site of drug release [14]; and fourthly, they are "Generally Recognized as Safe (GRAS)". This safety classification reduces or completely eliminates excipient related adverse effects or hypersensitivities observed in some polymeric nanosystems [15-17] . Thus, CyDs are considered ideal drug delivery carriers for filling the current gaps in the oral drug delivery paradigm.

Currently, considerable research attention is focused on the use of nanotechnology to construct various CyDs structural architectures tailored for optimum drug loading and superior bio-functionalities. The broad approach is to either entrap CyD-drug complexes in simple to intricate polymeric nanoparticles; or to directly construct nanoparticles from CyDs and CyD-polymer systems [10,18]. In addition, the past few years have witnessed a surge in available technologies and techniques for achieving these objectives. While many of the strategies are by and large innovative combinations CyDs and various polymers systems; others have focused on the use of both new and relatively old technologies to achieve previously tried objectives. Thus, this mini-review seeks to appraise the last two decades of CyD nanosystem development for oral drug delivery. It will first of all discuss the physiological basis of oral nanoparticle delivery in order to highlight current trends in the quest to control the gastric and *in vivo* fate of small molecular drugs and biopharmaceuticals. Finally, the research conducted to date in interesting and increasingly popular classes of CyD

nanosystems will be summarized; and future perspectives for their clinical application will be discussed.

2. Pharmaceutical nanotechnology and oral drug delivery

The oral route is considered the most important for drug delivery due to advantages of dosing flexibility, versatility in dosage forms, ease of (self)administration, convenience in chronic therapy, wide patient acceptability and consequent therapeutic compliance [19-21]. Despite its utility, gastric insolubility/poor dissolution, inadequate membrane permeability, and pre-systemic inactivation associated with many APIs continue to preclude optimal bioavailability, biodistribution and therapeutic efficacy [22]. It is currently estimated that about 40% and 90% of currently marketed drugs and those in developmental phase respectively, are plagued by these problems [23]. While many strategies have been advanced to address one or more of these challenges [24-27]; the nanotechnology-based approach offers an adaptive model for combining material science and various particulate technologies, with our understanding of the critical physicochemical and biological parameters that modulate oral drug bioavailability and biodistribution.

Three major absorption pathways of oral nanosystems (Figure 1.2.2) have been extensively discussed in literature. The first is the lymphatic pathway modulated by the Peyer's patches of the Gut Associated Lymphoid Tissues (GALT) [28]. These Peyer's patches, characterized by M-cells, are mostly located in the ileum [29]; and are specialized for continuous uptake of materials from the lumen by endocytosis. These materials are subsequently transported into intraepithelial spaces and underlying lymphoid tissues [30]. Nanosystems designed for lymphatic uptake are able to deliver the drug directly into systemic circulation by circumventing the liver and avoiding first pass metabolism [31,32]. Also, M-cells are relatively less protected by mucus secretions and drug efflux transporters like P-glycoproteins (P-gp); and as such, can serve as an important route for enhancing the pharmacokinetic profiles of their substrates [33,34]. The role of the lymphatic pathway in the transformation of dietary lipids (e.g. triglycerides) into colloidal lipoproteins and the subsequent shuttling of these materials through the mesenteric lymph vessels into systemic circulation has been basis for the development of numerous lipid nanocarriers, and triglyceride mimicking lipid prodrugs [35,36].

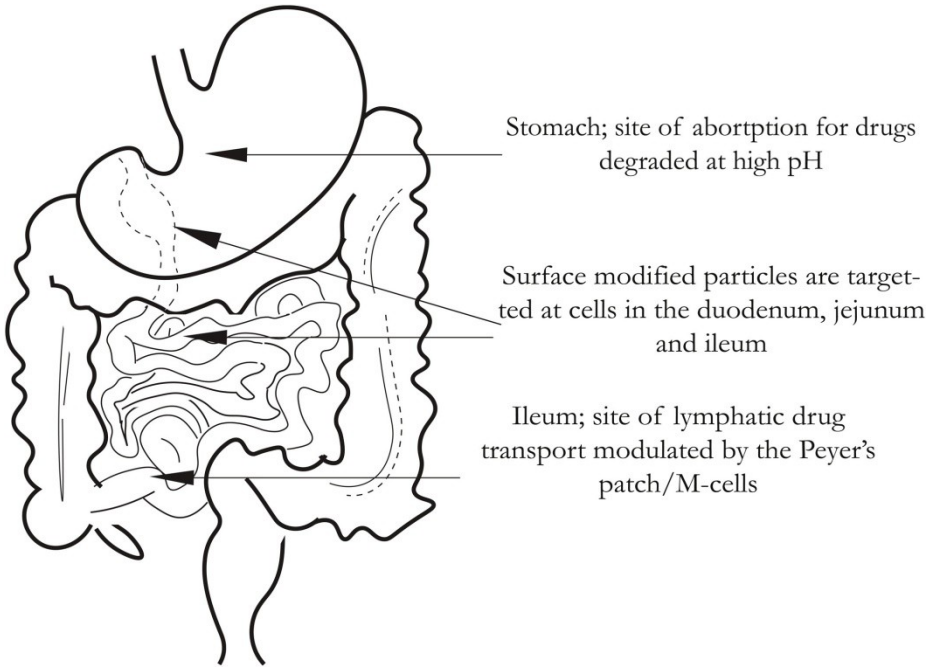

Figure 1.2.2: Important sites of nanosystem absorption in the gastrointestinal tract

The second approach focuses on transepithelial transport by modifying and/or functionalizing nanoparticle surfaces to actively to interact with specific cells capable of modulating residence time and/or uptake in the small intestine [31]. For instance, vitamin B-12 Intrinsic Factor (B 12-IF) expressed on the enterocytes of the duodenum, jejunum, and ileum, is responsible for the receptor-mediated transport of vitamin B-12 (cobalamin) through the lysosomal pathway [37,38]. Thus, surface modification or functionalization of an oral drug nanosystem with vitamin B-12 has been demonstrated to enhance drug uptake in the intestines of rats, dogs, and pigs; compared to unmodified nanosystems [38]. Similarly, coating nanoparticles with hydrophilic polyethylene glycol (PEG) has been used to enhance mucus layer penetration, thus bringing the nanosystem into close proximity of enterocytes on the epithelial surface of the small intestine [22,31]. Other cells present in the small intestine such as the goblet cells and the neonatal Fc receptors have also been exploited to enhance intestinal uptake of small molecule drugs and biopharmaceuticals from nanosystems [39,40].

Thirdly, drugs readily degraded by the high pH environment of the intestine (e.g. captopril) and those that exhibit a low pH-dependent absorption (e.g. verapamil and metronidazole) have been targeted to the stomach [31]. Such systems are required to withstand gastric peristalsis and their effectiveness is modulated by the gastric motility phase in play when the drug is swallowed. While mucoadhesive nanosystems can be used to prevent gastric emptying, the balance between mucosal adhesion and penetration is important for adequate

drug-epithelium interaction and enterocytes mediated transport [31,41]. Some nano-therapeutic approaches for treating GIT disorders such as *Helicobacter pylori* (H. pylori) infections and gastric ulcer have explored site-specific drug delivery at the site of injury [41-43].

Overall, the design of an effective oral nanosystem targeting any of these pathways relies heavily on the knowledge of the clinical requirements and biochemical parameters modulating the pharmacokinetic profile of the drug of interest; to enable a deductive manipulation of its physicochemical properties for efficient delivery and biochemical interaction *in vivo* [28,31,44,45]. Particle size, surface properties, molecular weight, hydrophobicity, and particle charge have all been demonstrated as important and tunable physicochemical properties for manipulating the clinical applicability of oral nanosystems [28,46-52].

3. Cyclodextrin-based nanosystems for oral drug delivery

3.1. Cyclodextrin nanoassemblies and supramolecular systems

Advances in supramolecular chemistry have enabled the construction of various biocompatible CyDs based nanosystems for a wide range of biomedical and drug delivery purposes. Many amphiphilic CyDs, CyD-polymer conjugate systems, CyD-polyrotaxanes etc. have been synthesized with their structure-property relationships and functionalities tailored by varying parameters such as the number, type, and position of the conjugates used [11,53,54]. The conjugated polymers or functional groups are grafted by enzymatic or chemical processes to the reactive hydroxyl groups (primary or secondary) of the CyD. Their self-assembling abilities are then mediated by electrostatic forces, hydrogen bonding, van der Waals attractions and host-guest interactions [55]. Generally, the classification of CyDs nanoassemblies and supramolecular systems using structural architectures (Figure 1.2.3) such as micelles, uni/multilamellar vesicles, nanospheres, nanocapsules, nanogels, nanoreservoirs, CyDplexes (Figure 1.2.3), etc. has been used to clarify specific physicochemical and drug delivery functionalities, especially as it pertains to drug loading mechanisms, particle size, morphology and surface properties. Individually, these structural architectures have also been prepared with non-CyD polymers and continue to be the subject of extensive research. However, by using a polymer perspective, this section reviews the synthesis, properties and oral drug delivery possibilities of several CyD nanoassemblies and supramolecular systems for diverse types of APIs delivery needs.

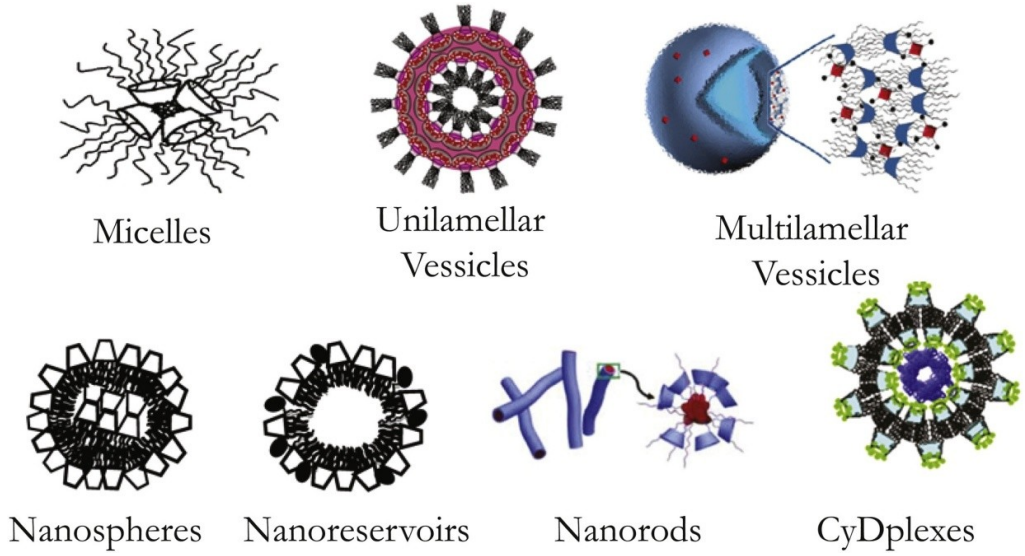

Figure 1.2.3: Schematic representation of some cyclodextrin nanoassemblies and supramolecular architectures [56].
Reprinted with permission from American Chemical Society for [57-61].

3.1.1. Cyclodextrin self-assemblies

Parent CyD molecules, their derivatives, and drug-CyD complexes behave like dispersed nanosystems by self-assembling in aqueous systems [62-65]. The aggregation behaviour of CyDs molecules and their drug complexes in aqueous solutions have been demonstrated using various measuring techniques such as; light scattering, electron microscopy, spectroscopy and osmolality [62,66]. For parent CyD molecules and their derivatives, this aggregation potential is generally weak with only ≈ 1% of dissolved parent CyDs able to aggregate into a size range of 60 - 120 nm [63]. While a higher self-assembling ability and a micellar-type aggregate formation with a particle size range of 380-750 nm have been observed with their inclusion complexes, the presence of a constant equilibrium (continuous aggregate formation and dissociation) between molecules forming the drug-CyD complex, and the possibility for both inclusion and non-inclusion complexes to co-exist are largely responsible for observed flux, instability and variations in structural architectures and properties [62,67,68]. Generally, this aggregation behaviour is self-mediated, dependent on the concentration of the drug-CyD complex i.e. it increases with increasing complexation [63].

Overall, a deep understanding of the mechanisms, aggregate properties (e.g. size and shapes) and how they can be controlled is only just evolving and further improvements in analytical

techniques will continue to elucidate technical knowledge and how these nanosystems can be optimally controlled and/or manipulated for efficient drug delivery [62,66]. Interestingly, some studies using hydrocortisone (aggregate diameter < 200 nm) [69], diflunisal [70] and ibuprofen [70] have already shown that drug CyD nanoassemblies can enhance desirable oral drug delivery properties like aqueous solubility and gastric permeability. Currently, a significant number of research groups are focused on the development and application of these nanosystems as a new breed of drug delivery nanosystems [62].

3.1.2. Amphiphilic cyclodextrin nanosystems

Amphiphilic CyDs, with hydrophilic head and hydrophobic tails groups are one of the most studied CyD molecules for non-covalent self assembling abilities. They can be directly prepared from CyD without the use of additional polymers or surfactants, and their versatility allows for the incorporation of both hydrophilic and hydrophobic drug molecules [56,71]. The general synthetic approach for amphiphilic CyDs is the controlled grafting of anchors like phospholipidyl, peptidolipidyl cholesteryl, oligoethylene (oxides) etc. on the hydroxyl groups of the CyDs molecules. Over the years, various cationic, anionic and neutral CyD amphiphiles have been prepared by varying the type of anchors used, their positions on the CyD molecule, and the method of preparation; to produce CyD nanoassemblies of varying structural architectures, physicochemical and drug delivery properties [11,72-74]. Amphiphilic CyDs are able to load drug molecules into three distinct domains of their nanoassembly, that is, the "aqueous" core of the nanoassembly, the lipophilic exterior and the hydrophobic cavity of the CyD [72]. In their study, Bilensoy and co-workers [75] have shown that a higher drug entrapment efficiency (compared to polymeric nanoparticles) can be achieved by drug incorporation into both the CyD cavity and the aliphatic chains of the amphiphilic CyD. They also observed a higher mucus penetrating ability of 6-OCAPRO-β-CyD amphiphilic nanocapsules compared to poly-ε-caprolactone based polymeric nanocapsules suggesting an enhanced ability to interact with biological membranes [75] . By using fluoroalkyl ester amphiphilic CyDs, a 5.8 h increase in the duration of antihypertensive effect of molsodomine was observed after oral administration, thus illustrating the ability of amphiphilic CyDs to facilitate sustained drug delivery and eliminate multiple (3-4 times per day) administration [76].

Furthermore, the potential application of these nanosystems for oral gene delivery especially in the treatment of local intestinal diseases like colon cancer and inflammatory bowel

diseases have been shown by their ability to transfect intestinal cells. Compared to commonly used polyethyleneimine, complexes of pDNA with poly-6-cationic amphiphilic CyD significantly increased pDNA uptake and gene expression in both undifferentiated and differentiated Caco-2 cells [77]. Interestingly, some research groups have recently explored the possibility of using hydrophobic APIs as anchors for synthesis of amphiphilic CyDs. Here, the CyD molecules act as the hydrophilic head while the drug is the hydrophobic tail of the nanoassembly. The disruption of the drug-CyD amphiphilic nanoassembly by an external agent or an *in vivo* stimuli can then lead to the desired delivery of the API (i.e. separation of drug from CyD) [78-80]. This could open up a new vista of possibilities where the complexity and undesired effects introduced by grafted anchors can be successfully eliminated.

3.1.3. Cyclodextrin-polymer conjugates

Cyclodextrin polymer conjugates covers a broad range of supramolecular systems where CyD molecules are either crosslinked to polymers leading to their incorporation in the main chain of final drug delivery nanosystem; or covalently conjugated to desirable polymers (Table 1.2.1) [11] . While many of these systems have been known to scientists for more than four decades, they have enjoyed renewed research interests since the turn of the millennium due to their potentials as building blocks for nanoparticulate drug delivery systems. Because of their huge number and overlap with other categories of CyDs nanosystems, it is almost impossible to describe or classify all the CyD-polymer systems that have been reported in literature. Here, a few examples are cited to highlight the broad strategy being employed and their oral drug delivery applications.

A series of studies pioneered by Prof Dominique Duchêne and co-workers in the late 90s were designed to address the drug loading problem associated with many polymeric nanoparticles by using CyD and a polymer as co-monomers of a final drug delivery polymer [81]. By simply preparing the polymeric nanoparticle in presence of a suitable CyD molecule, a non covalent interaction between the co-monomers (acrylic polymers were used) resulted in an increase in nanoparticle drug loading capacity [82-84]. Typically, the localization of the CyD on the CyD-polymer nanoparticle mediates their drug loading properties. While some of the CyD molecules are adsorbed on the nanoparticle surface from where they modulate surface properties and form inclusion complexes with guest molecules; others are entrapped within the polymer or incorporated into the polymer chain [81,85].

Table 1.2.1: Examples of synthetic routes for CyD-polymer conjugates (adapted from [66]).

Type of synthesis	Reagent	Substrate(s)	Description
Cross-linking	Epichlorohydrin, diepoxides, dissocyanates, diglicidylethers	CyDs and CyDs derivatives	One-pot method to tailor CyD polymers using low temperature, polycondensation can be stopped by adding acetone
		CyDs with polymers	
	CyDs	Polymers	
	End-modified polymers	CyDs	
Copolymerization	Vinyl- or (methyl)acryoyl-modified CyD monomers	Commonly used vinyl monomers (e.g. acrylic acid)	Chemically or radiation induced polymerization resulting statistical copolymers
Click chemistry	Azide-functionalized CyDs	Alkyne-modified polymers	Possibility to create open-chain CyD polymers with [1,3] cycloaddition
	Azide-functionalized polymer	Alkyne-modified CyD	
Schiff-base forming	CyDs with formyl group	Polymers with amine group (chitosan)	A way to prepare open-chain chitosan based CyD derivatives

In another study using the antiretroviral drug saquinavir, the authors demonstrated a CyD mediated reduction in the cytotoxicity of the acrylic polymer and an increase in the rate of drug transport, even though net bioavailability was not improved due to the inability of CyD to protect the drug from P-gp efflux transporters [86,87]. Over the last decade, this approach has been used to confer higher drug recognition capacity through multiple binding sites, especially for high molecular weight drugs and macromolecules [11]. A few examples of this

type of CyD-polymer system includes CyD-methacrylate [88,89], CyD-epichlorohydrin [90], CyD-dextrans [91,92], CyD-chitosan[93] etc.

Another variant of CyD-polymer systems is the encapsulation of pre-formulated drug-CyD complexes within polymeric nanoparticles [94]. This has been reported to allow for the inhibition of P-gp and cytochrome P450's (CYP P450) effect of APIs. For instance, Irache and co-workers reported a higher gastric permeability and bioavailability of paclitaxel, a substrate of P-gp and CYP 450, when its CyD inclusion complexes were incorporated into poly(anhydride) nanoparticles [95,96]. Cyclodextrins ability to protect APIs for gastric inactivation is particularly important for the oral delivery of biopharmaceuticals. In a study by Zhang et al. [97], epichlorohydrin and β-CyD nanosystem was prepared by poly-condensation, subsequently used to form insulin complexes. The insulin complex was then further encapsulated in alginate/chitosan nanoparticles. Drug release studies in simulated intestinal fluid showed higher insulin association efficiency and release in formulations containing CyD-epichlorohydrin. The retention of insulin in the nanoparticle core (absent on alginate/chitosan nanoparticles) was suggested to be responsible for the preservation of insulin structure, its protection from GIT degradation, and more than 2-fold increase in amount of insulin released [97]. Many more studies have demonstrated the ability of these CyD-polymer nanosystems to stabilize biopharmaceuticals within nanoparticles, protect the payload from gastric inactivation, and modulate their efficient [85,98-100].

Apart from the large library of homo-polymers available for CyD-polymer conjugates; multiple polymer systems [101] or block copolymers are also being used for the construction of these nanosystems. A recent study by Hu et al. [102] described polymer vesicles (polymersomes) based on β-CyD centered triarm star polymers (methoxy poly(ethylene glycol)-*b*-polylactide) developed to alter the absorption pathway of the chemotherapeutic agent doxorubicin. Although doxorubicin is a highly soluble class III drug according to Biopharmaceutics Classification System [103], its oral absorption is limited by poor permeability through paracellular drug transport. Thus, β-CyD mediated an increase in drug loading into the nanosystem, while the nanoassembly was able to alter the route of gastric uptake from paracellular to active transcellular transport. The observed seven and eight fold increase in bioavailability and half life of doxorubicin *in vivo* was correlated to the increase in the chemotherapeutic efficacy against a mouse sarcoma model. The formulation was also able to prevent cardiac and gastrointestinal toxic effects associated with doxorubicin [102].

Another distinct class of CyD-polymer conjugates systems are the polyrotaxanes and polypseudorotaxanes; a group of biodegradable polymers that are systematically assembled by threading multiple CyD molecules on polymer chains with or without bulky end caps [11,104]. Covalent bonds are absent and the presence of multiple hydroxyl groups in the final polymer allows for conjugation of a broad range of therapeutic agents in large doses. In addition, the presence of multiple CyD cavities allows for cooperative substrate binding especially when the inclusion complex formation ability of parent CyDs or their derivatives is limited [11]. They are particularly suitable for oral gene delivery due to the mobility of the threaded CyD molecules along the polymeric chain [105,106]. Some research approaches have been directed towards the construction of pH sensitive polyrotaxanes and polypseudorotaxanes; and their surface modification to enable controlled oral delivery applications [107].

3.2. Cyclodextrin nanosponges (CyD-NS)

Cyclodextrin nanosponges are cross-linked, hyper-branched, highly porous, crystalline or amorphous non-aggregating CyD-polymer nanosystems (Figure 1.2.4) [18,108,109] . Initially developed in 1998 for water purification [110,111]; CyD-NS have emerged over the last decade as a versatile and biocompatible nanosystem for oral drug delivery [23,109]. They are generally prepared by simple polymerization reaction between primary CyD molecules and bifunctional agents such as carbonyl compounds and organic dianhydrides [23,109,112].

Two major techniques; solvent evaporation and ultrasound assisted synthesis have been advanced for the synthesis of CyD-NS. In the solvent evaporation method, CyD molecule is dissolved in appropriate solvents (dimethylformamide, dimethylacetamide, dimethylsulfoxide, etc.) and reacted (a reaction initiator is often used) with an excess amount of the cross-linker. The resulting product is then recovered and purified by soxhlet extraction with ethanol or acetone. Ultrasound assisted synthesis [113] is a solvent-free method based on the sound induced cavitation of CyD and cross-linker at high temperatures (*ca*. 90 ^{o}C), and it is also followed by the removal of unreacted materials or undesired residues by purification. Some studies have reported other solvent-free methods such as heating under reflux and melting [114]. The reaction occurs through a nucleophilic attack on reactive OH-groups of the CyD molecules leading to the substitution of the hydrogen atom with functional groups from the cross-linking polymer. The mesh-like structural arrangement of the cross-linked groups in addition to the CyD cavities acts as pores or nanodomains for the

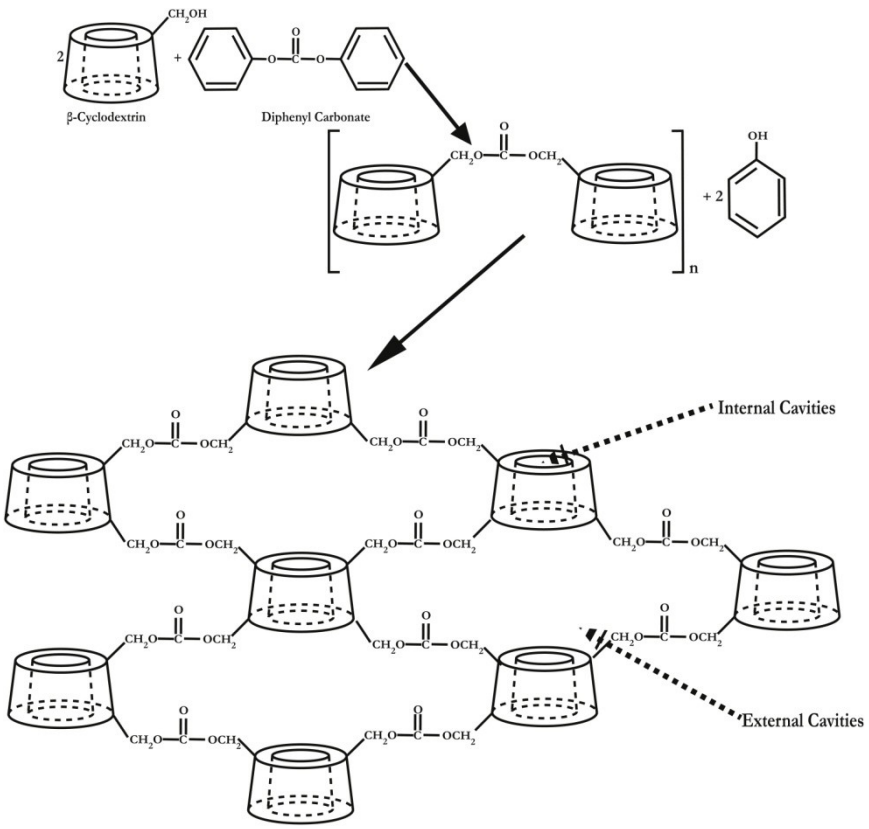

Figure 1.2.4: Schematic representation of cyclodextrin nanosponges prepared with β-cyclodextrin and diphenyl carbonate [23].

incorporation of nano-sized hydrophilic and hydrophobic drug moieties and the formation of both inclusion and non-inclusion complexes [108,114,115].The physicochemical and functional properties CyD-NS are usually controlled by the choice and ratio of cross-linking polymer, its molecular ratio to CyD; and also by the synthetic method used in its preparation [109]. CyD nanosponges are nontoxic and stable at high temperatures (up to *ca.* 300 °C) and insoluble in both water and organic solvents. Their capacity for high drug loading is particularly advantageous compared to some other drug delivery nanosystems and they can be easily tailored for selective drug release.

In oral drug delivery, CyD-NS are able to modify the physicochemical and pharmacokinetic profiles of active drug moieties by improving enhancing the apparent solubility of drug molecules in water. A Biopharmaceutical Classification System (BCS) class II drug, itraconazole with an aqueous solubility of about 1ng/ml showed a 27-fold increase in aqueous solubility when formulated with CyD-NS. The dissolution profile was also faster than that of the commercially available itraconazole, thus underlining the potential utility of CyD-NS to improve the pharmacokinetic profile of drugs whose bioavailability is limited by aqueous

solubility [116]. While the mechanism was not perfectly described, Torne et al, [117] enhanced the therapeutic efficacy of the anticancer drug tamoxifen with CyD-NS formulations. After oral administration by gastric intubation, the plasma concentration of tamoxifen-CyD-NS was significantly higher ($p < 0.01$) than that of the commercially available tamoxifen citrate originally developed to increase aqueous solubility and therapeutic efficacy of tamoxifen. Cytotoxicity studies on breast cancer MCF-7 cells also showed a concentration and incubation time-dependent inhibitory effect on cell proliferation, with CyD-NS formulation showing a better inhibitory effect.

Other studies have reported CyD-NS's ability to protect drug molecules from physicochemical degradation of APIs and its negative effect on therapeutic efficacy. Camptothecin, a potent anticancer agent was loaded into CyD-NS to explore the possibility of preventing degradation at physiological pH due to lactone ring opening and its reductive effect permeability through lipid bilayer [118]. Stability studies in phosphate buffer pH 7.4 and plasma showed the ability of CyD-NS formulation to protect the opening of the lactone ring by reducing the rate of hydrolysis and conversion of the drug to its carboxylate form instead of the more active lactone form [118]. Similarly, it has been suggested that CyD-NS might play a protective role against P-gp efflux transporters and CYP 450 enzymes to enhance drug permeability and subsequent bioavailability. Paclitaxel, a known substrate of CYP 450 3A and P-gp efflux pump in the epithelial cells of the small intestine and the liver [119]. The 2.5 fold increase in oral bioavailability of observed paclitaxel-CyD-NS formulation compared to the marketed Taxol® was suggested to be due to CyD-NS effect on the both solubilization and enzyme inhibition [120,121]. As with many other pharmaceutical excipients or delivery systems with gastric enzyme inhibitory ability; a clear elucidation of the mechanism of CYP P450 and/or P-gp inhibition and the quantification of the clinical significance of such activity will be important for knowledge based improvement of therapeutic outcomes of many drugs. Furthermore, the attenuation of adverse drug effect incidence and intensity, through the control of drug release and the potential application of CyD-NS for the delivery of proteins and peptides offers immense opportunity for the development of efficient drug delivery systems [122,123].

3.3. Electrospun Cyclodextrin nanofibers

Electrostatic forces and electrospinning have been used to produce synthetic filaments for about a century [124-126]. However, their more recent use in the production of nanometer

range fibers for various drug delivery and biomedical purpose has led to increased research attention over the past decade [125,127,128]. The potential of these nanosystems to modulate oral drug delivery has also been demonstrated using various drug delivery polymers [129,130]. Basically, nanofibers are produced when the electrostatic charge in the electric field (between a positively charged capillary filled with the polymer solution and a grounded target) overcomes the surface tension of the polymer solution to generate a polymer jet which travels to the grounded target as nano-sized fibers [131]. Nanofibers are highly porous multifunctional structures with large surface-to-volume ratio capable of delivering small molecular drugs and biopharmaceuticals [128,132].

Towards the end of the last decade, Tamer Uyar and his team [133] pioneered the use of CyDs in the preparation of electrospun nanofibers. Their first report was on the formation of nanofibers from α-CyD-PEG pseudopolyrotaxanes without the destruction of the inclusion complex and channel packing of their structure. Following the initial success, they functionalized polyethylene oxide (PEO) nanofibers with CyD molecules [134], prepared nanofibers from CyD polystyrene systems [135] and eventually produced nanofibers from CyD molecules alone [136]. Using Methyl-β-CyD alone, they demonstrated the dependence of the produced nanofibers on the initial concentration of CyD solutions. A high CyD concentration was found to facilitate significant intra-molecular reactions and chain entanglement between CyD molecules, thus maintaining continuity of the jet during the electrospinning process and avoiding the formation of beads due to electrospraying. An increment in fiber diameter range (nm) was also observed when changing the solvent system from water to *N,N*-dimethylformamide [136,137]. While CyD nanofibers are able to entrap various drug molecules, electrospinning CyD inclusion complexes have also been carried out to modify drug release properties and to enhance *in vivo* functionality [137,138]. Despite these potentials, a review of literature suggests that the use of CyD based nanofibers for the oral drug delivery is still largely focused on CyD-polymer hybrid systems.

For instance, Canbolat et al. [139] reported a two-fold increase in drug release with nanofibers of naproxen-β-CyD inclusion complex in polycaprolactone compared to naproxen alone. Manasco et al. [140] elucidated the relationship between the drug release profile and CyD content of the CyD-polymer nanofiber system. They observed that nanofibers with higher HP-β-CyD content quickly released most drug content and that drug loading and dissolution rate can be adequately tuned by varying the HP-β-CyD/PVA (polyvinyl alcohol) ratio. By improving active drug dissolution, nanofibers have become attractive for the

development of orally disintegrating dosage forms. These dosage forms are usually prepared for pediatric and geriatric patients with compromised swallowing ability (tablets and capsules) due to various physiological and psychological factors [141]. A fast oral disintegration and taste masking ability have been reported for a CyD-PVP (polyvinylpyrrolidone) nanofiber system of meloxicam [142]. Interestingly, another study by Vigh et al. [143] has shown the suitability of a CyD (alone) nanofiber system for orally dissolving formulations by achieving a near total dissolution and drug release of spironolactone. The potential for enhancing therapeutic efficacy by incorporating CyD into electrospun polymer nanofiber systems has also been demonstrated in a study comparing silver nanoparticles of CyD/PVA nanofibers to those of PVA nanofibers alone [144]. Although this study was not designed for oral delivery, the nanofiber formulation enhanced the control of particle size and the antimicrobial/therapeutic activity applications of silver nanoparticles [145,146]. By functionalizing these systems to achieve stimuli-responsive or site-specific drug release, the pharmacokinetic profile of many orally administered drugs can also be improved[147] .

3.4. Cyclodextrin molecular imprints

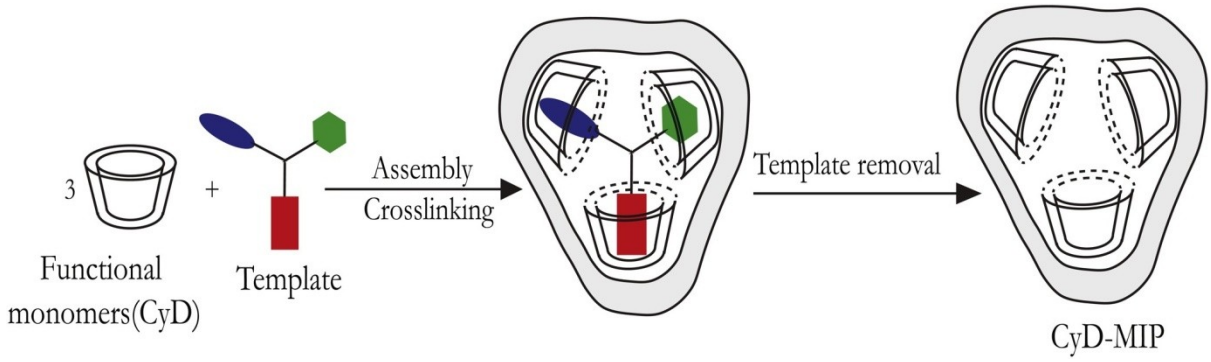

Figure 1.2.5; Cyclodextrin molecular imprinted polymers containing ordered assembly of three cyclodextrin monomers.

Molecular imprinting technology continues to enjoy considerable research focus as a synthetic method of developing molecular affinity systems that are able to recognize, form complexes with, and control the delivery of small molecular drugs and biopharmaceuticals. Molecular imprinting involves the design of polymer matrices that have specific and complimentary recognition/binding sites for an active drug molecule (called template) [148,149]. The spatial orientation of the functional monomers is fixed during the imprinting

process thus allowing the polymer matrix to retain the conferred molecular recognition capacity (Figure 1.2.5) [150]. By arranging the interacting group of the functional monomers, the molecular structure of the polymer matrix are specifically adapted to the template of interest, leading to a higher drug loading efficiency [151]. Oral drug delivery applications of molecular imprinted polymers (MIP) includes the protection of the template from degradation triggers in the GIT and the modulation/control of the drug release and pharmacokinetic profile [149]. Functional response to an external stimulus such as pH, temperature, presence or absence of chemical species etc., and the possibility to tailor both drug recognition and delivery properties continues to make MIPs attractive for oral drug delivery [152].

Cyclodextrin molecules are important functional monomers for MIP synthesis due to their capacity for hydrophobic effect-based recognition in an aqueous environment. By using more than one CyD molecule, an ordered assembly of multiple monomers can be produced with each monomer selectively binding a portion of the template (Figure 1.2.5) [153]. This confers the ability to form complexes with high molecular weight APIs [153]. Asanuma et al. [154] developed a CyD-MIP network with the CyD molecules spatially arranged to fit designated portions of a nano-scaled vancomycin, an antibiotic glycopeptide with a limited oral administration [155,156]. Compared to the polymer matrix without recognition capacity, a 2-fold increase in drug loading was observed in the CyD-MIP systems. Also, a 2.6 fold increase in the apparent stability constant suggested that the CyD-MIP is able to achieve sustained release of the active drug [154,157]. Recently, MIPs of CyD-NS were prepared by Trotta and co-workers for oral delivery of L-DOPA in the treatment of neurodegenerative diseases . Theoretically, the synthesis of CyD-NS in the presence of the template alters their structural arrangement, molecular recognition ability, and *in vivo* behavior; compared to drug loaded CyD-NS. The prolonged rate of L-DOPA release from MIP-CyD-NS compared to non-imprinted CyD-NS suggested a stronger, molecular imprinting mediated L-DOPA-CyD-NS interaction [158].

It is important to note that despite their potentials, CyD-MIPs are still grossly understudied for drug delivery compared to separation science and water treatment. According to Silva and Casimiro, [152] out of about 13,000 papers published in molecular imprinting up to 2012, less than 170 were related to drug delivery. A significantly less amount utilized CyD molecules. Thus, increased research activity is required if prospects of these nanosystems will be harnessed in overcoming the biological and physicochemical impediments to optimum therapeutic efficacy.

4. Conclusion and future perspectives

The appeal of pharmaceutical nanotechnology and its potential for the development of translational and innovative therapies are indisputable. By offering immense possibilities for achieving desired control over the *in vivo* fate of small molecule drugs and biopharmaceuticals, it is perhaps safe to conclude that the future of drug delivery is firmly rooted in nanomedicine.

The application of nanotechnology in the development of efficient (pharmacokinetic), low-cost and safe oral drug delivery systems is primarily driven by specific drug delivery needs of many clinically available and new chemical entities, and the advantage of convenient therapy provided by oral medications in life-long drug use associated with many chronic diseases.
Cyclodextrin-based nanosystems are being increasingly studied and are emerging as promising platforms for providing pharmacokinetic and formulation design efficiency, without a compromise on safety. This review has identified and illustrated various important strategies that have become popular over the past two decades and are currently being exploited by many research groups to construct and optimize CyD nanosystems for oral drug delivery.

The ease of CyD modification and supramolecular systems construction is perhaps responsible for the high research interest and the influx of many CyD based nanosystems into science literature. These nanosystems are being constructed and manipulated to meet drug specific oral therapeutic needs due to their wide range of physicochemical and biological properties and their ability to provide comparative drug delivery advantages over other polymeric nanosystems. Already, desirable oral drug delivery system properties like high drug loading, gastro-stability, GIT site-specific delivery, controlled and targeted drug release, non-immunogenicity/toxicity and biodegradability have all been demonstrated by many researchers working with CyD nanosystems. Despite the potentials and reported advantages, many of these systems have not yet been fully exploited for clinical purposes. This is partly due to the fact that many research papers do not provide a full description of the mechanisms by which these nanosystems modulate oral drug delivery and the specific role of cyclodextrin in such modulations. Superior pharmacokinetic behavior and comparative analysis are often used as a proof of utility and to "speculate" their mechanisms of action. Also, many of the new technologies being used in the construction of CyD nanosystems are not yet easily scalable for industrial manufacturing and often introduce more complexity into these systems.

These complexities may be reduced by identifying specific system-property relationships of these systems to enable their simplification. Also, while there is still a broad range of diseases requiring innovative nanotechnology-based therapies, it is also important that research focus is not limited or restricted to pharmacokinetic and potential therapeutic benefits alone. More studies looking to clarify the large scale manufacturability, regulatory issues and safety concerns are required for adequate clinical exploitation of these systems.

As already observed with many other nanoformulations, the inability to translate many laboratory efficient and promising pre-clinical nanosystems into clinically useful products remains a problem. The slight increase in *in vivo* pharmacokinetic and therapeutic performances commonly observed in studies attempting to optimize clinically available drugs with nanosystems are often inadequate to provide a cost-performance benefit. Also, the non-human nature of many models used in the pharmacokinetic evaluation of these oral nanosystems contributes to their high attrition rate of many nanosystems in clinical trials. Recent advances in the development of models with increasing ability to perfectly simulate and/or predict drug pathway through the GIT into the systemic circulation by mimicking relevant human physiological conditions are expected to improve the clinical translation of these systems.

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Section 3

HIV/AIDS: Description, pharmacotherapy and drug delivery strategies

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1 Human Immunodeficiency Virus (HIV): Description and epidemiology

Infection with Human Immunodeficiency Virus type 1 (HIV-1) and type 2 (HIV-2), which were first isolated in 1983 and 1985 respectively, is of zoonotic origin from African primates (simian immunodeficiency virus) and is responsible for causing numerous opportunistic infections, rare malignancies and co-morbidities referred to as Acquired Immunodeficiency Syndrome (AIDS)[1-5]. Permanent human infection with HIV occurs through the post-exposure entry and subsequent integration of the viral genome into host cells. For entry into the cells, the viral glycoprotein (gp120) binds to $CD4^+$ (HIV receptor) and either of the two chemokine receptors, CCR5 and CXCR4 on cell membranes, while the trans-membrane glycoprotein gp41 modulates the viral fusion into such cells (Figure 1.3.1) [6,7]. The post entry integration allows for viral replication, continuous depletion of $CD4^+$ T_H cells (T-Helper lymphocytes), subsequent immune suppression and progression of the infection to AIDS [8].

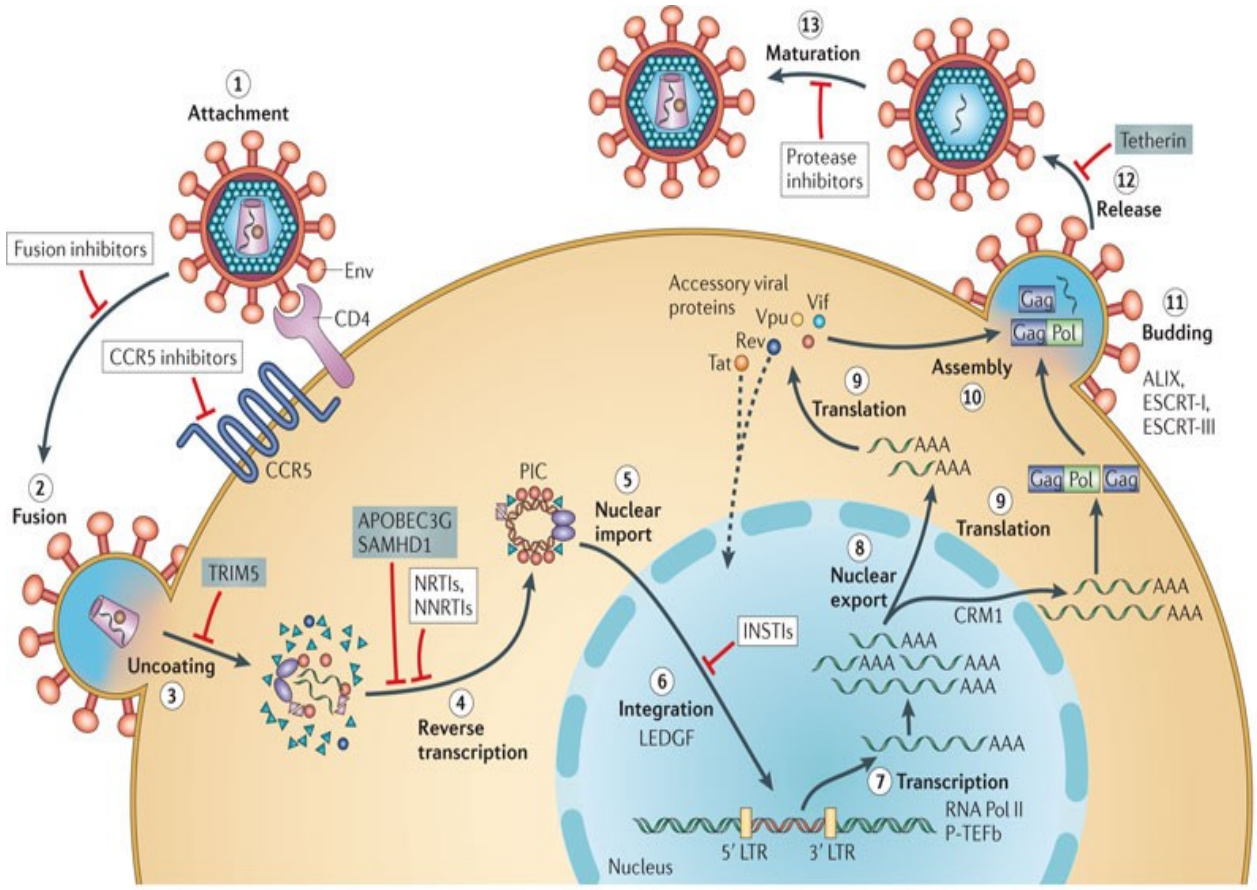

Figure 1.3.1: Schematic overview of HIV infection and targets of drug action [7]

Over a period of three and a half decades, HIV/AIDS has resulted in the death of 39 million people [9]. In 2015 alone, about 1.2 million people died from HIV related causes [9]. Currently, about 37 million people are infected with the virus, with an estimated rate of 2.1 million new cases per annum [9-12]. While a lot of success has been achieved in the science of HIV diagnosis, treatment and health care policy development; more needs to be done if the global objectives for ending this pandemic are to be met by 2030 [13-15]. Global prognosis for HIV pandemic remains unpropitious due to continuous spread of the infection, problems associated with drug resistance, acute/chronic side effects of drug therapy; and the inability to restore normal immune function by completely eradicating both long-lived latently infected cells and replication competent provirus from the body [11,13,16-18]. The successful treatment and/or potential cure of this global pandemic has so far been described as one of the insurmountable problems of the 21st century [10,19,20].

2 HIV pharmacotherapy, limitations and drug delivery strategies

Currently, there are seven classes of antiretroviral drugs (ARVs) and more than 28 drugs approved for the treatment of the HIV/AIDs [21,22]. These drugs are administered in combination as Antiretroviral Therapy (ART). The current therapeutic guideline (Table 1.3.1) includes regimens consisting of at least, two Nucleoside Reverse Transcriptase Inhibitors (NRTIs) and either a Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), a Protease Inhibitor (PIs) or an Integrase Strand Transfer Inhibitor (ISTI) [23-25]. The implementation of ART successfully leads to the control of viral replication and diseases progression, while allowing for concurrent repair of the immune system [26-28]. By suppressing viral load in people living with virus, HAART has reduced HIV/AIDS related mortality and morbidity, and also the risk of HIV transmission.

One of the targets of the global health sector strategy in ending HIV infection includes the optimization of treatment and care outcomes [29]. The need to optimize ARVs through better drug delivery strategies represents an important approach for enhancing therapeutic outcomes, reducing adverse drug effects and cost of therapy; and meeting the broader targets for HIV treatment [20,26,30-37]. It is no longer enough that ARVs are active in the prophylaxis of HIV, new methods and technologies that can optimize these drugs to enhance drug therapy outcomes are needed. This need is even more severe in the paediatric and adolescent population whose drug delivery needs have lagged behind those of adults due to

its complexities and time consuming nature of their development [37]. For instance, the paediatric oral solution of lopinavir (LPV) is currently formulated with 42 % $^{w}/_{v}$ of alcohol and 15.3%$^{w}/_{v}$ propylene glycol [38,39]. The high alcohol content of this formulation is clearly undesirable. Also, the recent recommendation from the START study (Strategic Timing of AntiRetroviral Treatment) to initiate ARVs in the 35 million people living with HIV [40,41] and their increasing role in pre-exposure prophylaxis [42,43] has reinvigorated the discourse on ARV drug optimization [23]. It is expected that by reformulating existing drugs and developing new drug delivery systems; bioavailability, biodistribution, tolerability and convenience can be enhanced; while dose related toxicity and inherent active pharmaceutical ingredient (API) cost can be reduced without a compromise on therapeutic efficacy [23,44-46].

Table 1.3.1: WHO recommended first-line ART regimen [25]

	Preferred first-line regimens	Alternative first-line regimens
Adults	TDF+ 3TC (or FTC) + EFV	AZT + 3TC + EFV(orNVP) TDF+ 3TC (or FTC) + EFV TDF+ 3TC (or FTC) + $EFV_{(400mg/day)}$ TDF+ 3TC (or FTC) + EFV
Pregnant or breastfeeding women	TDF+ 3TC (or FTC) + EFV	AZT + 3TC + EFV(orNVP) TDF+ 3TC (or FTC) + NVP
Adolescents	TDF+ 3TC (or FTC) + EFV	AZT + 3TC + EFV(orNVP) TDF (or ABC) + 3TC (or FTC) + DTG TDF (or ABC) + 3TC (or FTC) + $EFV_{(400mg/day)}$ TDF (or ABC) + 3TC (or FTC) + NVP
Children 3 years to less than 10 years	ABC + 3TC + EFV	ABC + 3TC + NVP AZT + 3TC + EFV (or NVP) TDF + 3TC (or FTC) + EFV (or NVP)
Children less than 3 years	ABC (orAZT) + 3TC + LPV/RTV	ABC (or AZT) + 3TC + NVP

3TC lamivudine, ABC abacavir, AZT zidovudine, DRV darunavir, DTG dolutegravir, EFV efavirenz, FTC emtricitabine, LPV lopinavir, NVP nevirapine, RTV ritonavir, TDF tenofovir.

Currently, almost all of the ARVs in clinical use are administered through the oral route of drug delivery. This offers amongst other advantages versatility in dosage forms, ease of (self)administration, and a wide patient acceptability that enhances drug adherence and the convenience required for chronic therapy [45,47-49]. Oral drug delivery is expected to remain important in the fight against HIV due to the volume and ambulatory nature of current

therapeutic interventions. However, despite their numerous advantages, the drawbacks of many commonly used ARVs continue to preclude optimal oral bioavailability, biodistribution and therapeutic efficacy [50,51]. Almost all ARVs of the NNRTI, PI and ISTI classes belong to Biopharmaceutical Classification System (BCS) class II and IV [52-55]. The BCS is used to classify drugs based on their oral aqueous solubility and permeability, and the effects of these parameters on their *in-vivo* efficacy [56,57]. Drugs belonging to the BCS class II have low aqueous solubility and high gastrointestinal permeability while class IV drugs have low solubility and low permeability.

Sosnik and Augustine [58] have summarised the key limitations to current ARV oral drug delivery systems and suggested practical strategies to resolving them. Over the past few years, ARV drug delivery research has focussed on enhancing gastric solubility, intestinal permeability and HIV reservoirs targeting. Also, a tremendous amount of research efforts is being expended in the development of long acting ARV delivery systems in an attempt to address the pill burden responsible for poor medication adherence [12]. Majority of the drug delivery strategies currently being studied includes the application of nanotechnology, exploitation of (non)polymeric excipients, optimization studies using design of experiments and various oral dosage form technologies to enhance oral solubility, permeability and HIV reservoir targeting [59,60].

3 Conclusion

Thus, the development of innovative oral drug delivery systems able to reproducibly provide precise therapeutic benefits that fills the current gap in HIV therapeutics will not only improve the prognosis of HIV, but also the socioeconomic factors associated with current treatment paradigm. While recent advancements in micro and nanoparticle based approaches are promising, to the best of our knowledge, no ARV delivery nanosystem or CyD based systems have been successfully developed for ARV therapeutics. Hence this study.

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CHAPTER 2

Preliminary Studies

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Section 1

Lopinavir-cyclodextrin complex formation: *In silico* and experimental evaluation of host-guest molecular interaction

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1. Introduction

The development of any cyclodextrin (CyD) based drug delivery system typically starts with an experimental evaluation of the stability constant (also known as equilibrium constant, formation constant, or association constant). This provides an insight into the strength of the molecular interaction/complex formation between CyD and the drug. CyD complex formation is usually described as an equilibrium between known molecules of the drug and those of CyD (Eq 1) [1] and several factors (e.g. polarity, steric dimensions, cavity size, electronic effects, temperature, pH, hydrogen bonding, van der Waals forces, surface tension, molar ratio etc.) have been reported to affect the nature and extent of complex formation [1-3].

$$K_{m:n} = \frac{[D_m\, CyD_n]}{[D]^m \times [CyD]^n} \qquad (1)$$

Where m and n are the number of drug (D) and CyD molecules respectively and $K_{m:n}$ is the stability constant

Since the most common stoichiometry of drug-CyD complexes is 1:1, stability constant is normally written as $K_{1:1}$. Experimentally, $K_{1:1}$ is determined by measuring CyD concentration dependent changes in the physicochemical properties (e.g. solubility, molar absorptivity, NMR shifts, etc) of the drug [3,4]. Its pharmaceutical application is primarily as a screening tool for the selection of the CyD molecule with the highest molecular affinity for the drug of interest [5]. Higher values of $K_{1:1}$ signifies a higher drug affinity for the CyD.

Despite its utility and popularity, the experimental limitations inherent in each of the possible methods for determining $K_{1:1}$ and its inability to provide a comprehensive understanding of the complex interacting phenomena mediating CyD complex formation remains an important constraint in their application. Over the past two decades, *in silico* approaches have become increasingly popular as useful tools for obtaining a mechanistic understanding of the CyD complex formation, and for avoiding or anticipating extensive laboratory experiments [6-9]. At the molecular level, detailed information relating to conformation, energy profile, solvation, recognition behaviour and function etc. between a CyD molecule and drug

can be obtained *in silico* [10-12]. These *in silico* methods allows for a rational, deductive and knowledge based approach to the development of CyD based drug delivery systems.

Herein, we report the development and experimental validation of an *in silico* method for the screening and selection of the CyD molecule with the best potential for developing a lopinavir (LPV)-CyD drug delivery system. Ibuprofen (IBU), a non-steroidal anti-inflammatory drug whose proven ability to form CyD inclusion complex has been previously reported [13-17] was used as a model drug to experimentally validate the *in silico* method. The validated method was then applied to LPV-CyD systems.

2. Experimental

2.1 Materials

LPV and IBU were gifts from Mylan (Hyderabad, India) and Laboratorios Medinfar (Portugal) respectively. Alpha-cyclodextrin (α-CyD) and Beta-cyclodextrin (β-CyD) were gifts from Wacker (Germany). Gamma cyclodextrin (γ-CyD, Cavamax) and (2-hydroxy)propyl-β-cyclodextrin (HP-β-CyD, HP Pharma) with degree of substitution, 0.6; were gifts from Wacker (Germany). Ethanol (96%) was purchased from Carlo Erba Reagents (Portugal), while purified water was obtained by inverse osmosis (Millipore, Elix 3). All reagents were used as received without the need for further purification.

2.2 Methods

2.2.1 Validation of In-silico and experimental methodology

Preliminary studies were carried out to test the hypothesis of using the molecular modelling approach to evaluate CyD host-guest interaction for the selection of the most optimal CyD molecule for the development of a new CyD based drug delivery system. For this purpose, IBU was selected as the model drug due to its proven ability to form inclusion complex with CyD. Phase solubility studies and molecular docking studies were carried out and the results were evaluated for rank order correlation.

2.2.1.1 Phase solubility studies

Phase solubility studies were carried out to study host-guest interactions between IBU and three CyD molecules (α-CyD, β-CyD and γ-CyD) as described by Higuchi and Connors [18]. Briefly, excess quantities of IBU (i.e. above its solubility) were added to increasing

concentrations of CyD aqueous solutions in capped tubes. The tubes were placed in a water bath at a constant temperature (25 °C ± 0.5), and were shaken in horizontally (100 movements/min) for a period of 8 h to allow for equilibration. Samples were collected and filtered (0.45 μm) prior to the quantification of the drug in solution by ultraviolet spectroscopy analysis (U-1900, Hitachi, Japan). All experiments were carried in triplicates and the mean values were used in the phase-solubility plots. Phase solubility curves were then plotted as the total dissolved drug concentration against the concentration of CyD. The apparent stability constant ($K_{1:1}$), complexation efficiency (CE) and drug : CyD molar ratio (D:CyD) were calculated from the slope of the straight-line portion of the curve, according to the Equations 2, 3 and 4 [4]:

$$K_{1:1} = \frac{Slope}{S_{int}(1-Slope)} \quad (2)$$

$$CE = S_o \,.\, K_{1:1} = \frac{[D/CyD]}{[CyD]} = \frac{Slope}{(1-Slope)} \quad (3)$$

$$D:CyD = 1:(1+\frac{1}{CE}) \quad (4)$$

Where, S_o and S_{int} are the intrinsic solubility and the solubility obtained from the intercept of the phase solubility plot respectively

2.2.1.2 In silico studies

Molecular docking studies were carried out to evaluate the possible binding interaction between IBU and three CyDs molecules (α-CyD, β-CyD and γ-CyD) using the Molecular Operating Environment software package (MOE, Chemical Computing Group). Briefly, the initial molecular geometry of IBU was obtained from PubChem database (CID 3672) while those of the parent CyD molecules were retrieved from the Protein Data Bank (2ZYM for α-CyD, 3CGT for β-CyD and 2ZYK for γ-CyD) and refined using the builder function on Molecular Operating Environment (MOE v2018.10, Chemical Computing Group, Canada). Prior to measuring the binding energy, these molecules were prepared by adding the hydrogen atoms at eh right pH and their energies were minimized using the MMFF94x force field. In this molecular docking program, IBU was set as the ligand while CyDs molecules were set as receptor. The flexibility of the ligand was considered in opposition to the

restriction of CyD flexibility which was defined as a rigid body. A maximum of 100 molecular ensembles of the ligand placed in the site with the Triangle Matcher method were saved and then ranked with the GBVI/WSA ΔG scoring function. The most energetically favourable binding poses were then chosen, the minimum energy mode of IBU, CyD and IBU-CyD complex were computed and the complexation energy was then calculated according to Eq. 5:

$$\Delta E = E_{complex} - \left(E_{host} + E_{guest}\right) \quad (5)$$

Where, ΔE, is the complexation energy and $E_{complex}$, E_{host} and E_{guest} are the minimum energy mode for the complex, CyD and IBU respectively

2.2.2 LPV-CyD complex formation: phase solubility and molecular docking studies

2.2.2.1 Phase solubility studies

Phase solubility studies were carried out by slightly altering the method described in section 2.2.1.1. Here, excess quantities of LPV were added to increasing concentrations of CyD 50% hydroethanolic solutions in capped tubes to allow for easy detection and quantification of the LPV in solution by UV spectrophotometry. To assess the trend in the solubilising/complexation effects of CyD on LPV, samples were removed from the tubes after 24, 48 and 72 hours, centrifuged at 10,000 rpm for 10 minutes prior to UV quantification and calculation of $K_{1:1}$, CE, and D:CyD as previously described.

2.2.2.2 *In silico* studies

This was also carried out as previously described section 2.2.1.2. In addition, the initial molecular geometry of LPV was obtained from PubChem database (CID 92727) while various derivatives of CyD were prepared from the parent molecules. Since it is impractical to simulate all possible isomers of substituted CyD, a set of 5 substitutions were simulated for each derivative on most reactive sites of glucopyranose unit, i.e., the secondary OH 2 and primary OH 6 groups [19,20] as shown in Table 2.1.1 below:

Table 2.1.1: Model summary for *in silico* derivatization of parent CyD molecules

CyD Derivative	Substitution	Models	Substitution position	
			OH_2	OH_6
(2-hydroxy)propyl-beta-CyD (HP-β-CyD)	$CH_2CHOHCH_3$	A	All	None
		B	All	All
		C	All	1,3,5,7
		D	1,3,5,7	1,3,5,7
		E	None	All
Sulfobutyl Ether Beta CyD (SBE-β-CyD)	$(CH_2)_4SO_2$	A	All	None
		B	1,3,5	2,4,6
		C	2,4,6	1,3,5
		D	1,3,5,7	2,4
		E	None	All
(2-hydroxy)propyl-gamma-CyD (HP-γ-CyD)	$CH_2CHOHCH_3$	A	All	None
		B	All	All
		C	All	1,3,5,7
		D	1,3,5,7	1,3,5,7
		E	None	All

3. Results and Discussion

3.1 Preliminary studies with Ibuprofen

The IBU-CyD phase solubility plots (Figure 2.1.1) were classified as B_S type for β-CyD and as A_L type for both α-CyD and γ-CyD respectively; using the Higuchi and Connors model [18]. The solubility of IBU increased to a maximum of 6 x 10^{-3} M, after which insoluble precipitation of both free and complex drugs began to occur with increase in β-CyD concentration. This type of relationship has been previously reported by Salustio *et al;* [17]. On the other hand, the solubility of IBU increased with an increasing concentration of both α-CyD and γ-CyD up to a maximum of 2.4 x 10^{-2} M. This A_L type relationship has been generally accepted to be indicative of 1:1 molecular ratio between the CyD and the drug molecule.

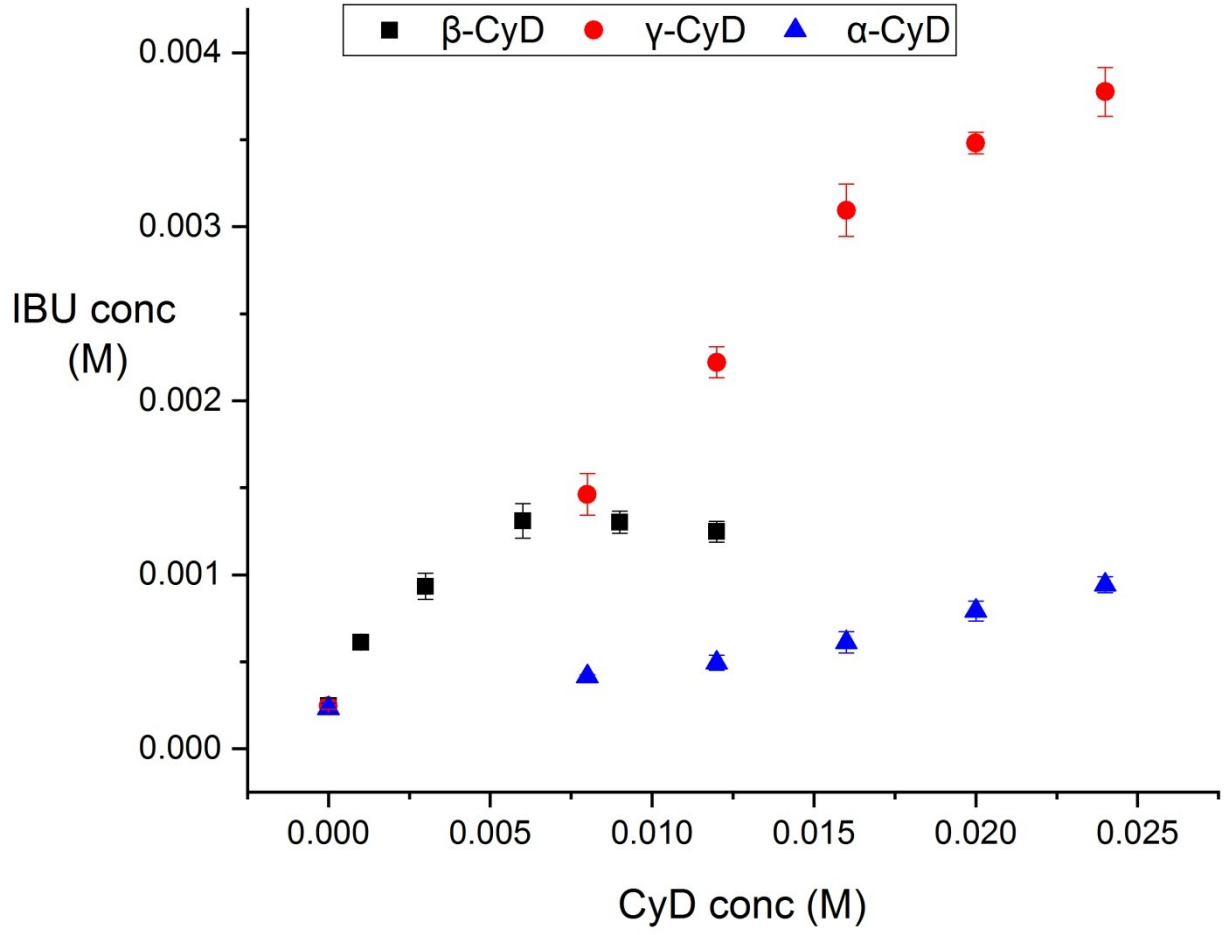


Figure 2.1.1: Phase solubility curves for IBU-CyD complexes

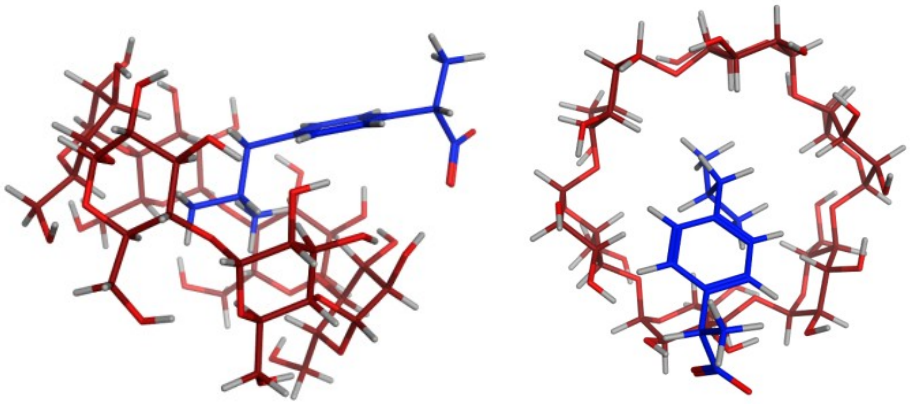
The $K_{1:1}$ calculated for the line equation of the phase solubility plots are also presented on Table 2.1.2. A slope less than the unity is generally accepted to be indicative of the formation of a 1:1 complex. The $K_{1:1}$ rank order of γ-CyD > β-CyD > α-CyD suggests the formation of stronger bond between γ-CyD and IBU. However, It is important to note the potential for erroneous $K_{1:1}$ values due to the dissimilarity between intrinsic solubility (S_o) and the theoretical solubility obtained from the intercept of phase solubility plots (S_{int}), especially for poorly soluble drugs (solubility < 0.1mM). This has led to the application of CE as a more precise (S_o and S_{int} independent) method of evaluating the solubilising effect of CyDs [4]. Also, other parameters that can be calculated from CE, such as D:CyD which can provide a more accurate estimation of molar ratio compared to those obtained from the visual inspection of the phase solubility plot type [21]. From Table 2.1.2, CE values for γ-CyD and β-CyD suggests slight differences in their ability to solubilise IBU while D:CyD values

points to molar ratios higher than 1:1. The visual evaluation of the phase solubility plots also suggests that this extrapolative methodology is unable to clearly characterise host guest interaction between several CyD molecules. For instance, the plot visually suggests that the use of the linear portion of the plot for β-CyD completely eliminates the role of intrinsic CyD solubility in the observed host-guest interaction. An almost equal solubilising and complexation effect for both γ-CyD and β-CyD will be concluded when the B_S nature of β-CyD plots and consequent IBU precipitation is excluded from the calculations. Thus, this necessitates the use of other analytical methods to further elucidate the true mechanism of interaction.

Table 2.1.2: Phase-solubility study data of IBU and CyD

CyD	Slope	S_o (Intercept)	R^2	K1:1	CE	D:CyD
α-CyD	0.0295	0.0002	0.973	151.984	0.030	33.898
β-CyD	0.1672	0.0004	0.955	501.921	0.201	5.981
γ -CyD	0.1682	0.0002	0.992	1011.060	0.202	5.945

Considering these limitations, it was hypothesised that the combination of an *in silico* method with the results of phase solubility studies should provide a more realistic idea of IBU-CyD interactions. It is important to note that despite their utility, different computational softwares and *in silico* methods for studying CyD host-guest interactions have often resulted in contradictory and conflicting results [22,23]. Thus, our approach was to compare the rank order of CE (calculated from the phase solubility plots) with the complexation energies (ΔE) (obtained from a simple molecular docking protocol carried out on MOE); and to evaluate the potential of using this *in silico* tool to rank LPV interaction with various CyD molecules. Figure 2.1.2 shows the optimal models of IBU-CyD complexes obtained from the docking study while Table 2.1.3 presents the docking score and complexation energy for IBU CyD interaction.



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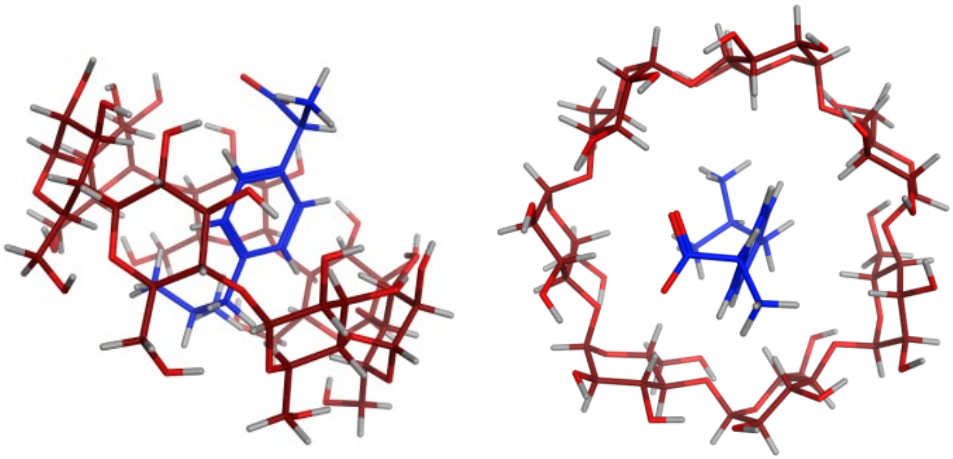

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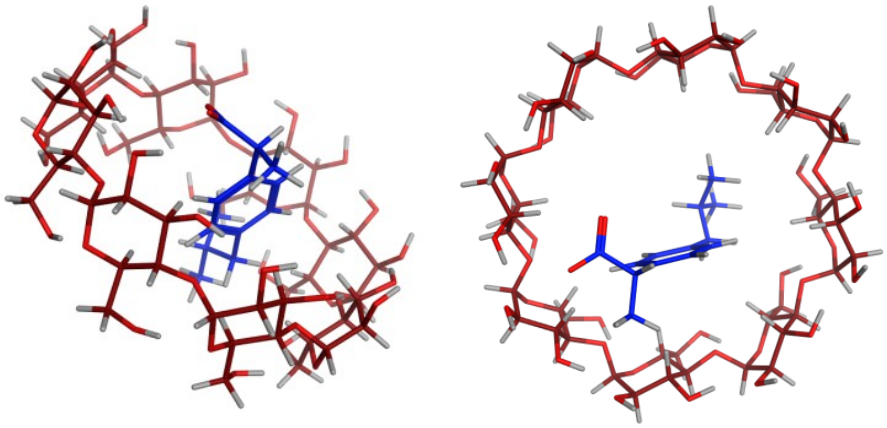


(c)

Figure 2.1.2: Optimal models of IBU-CyD complexes obtained for Molecular docking studies (a) IBU- α-CyD (b)IBU- β-CyD (c) IBU-γ-CyD

Table 2.1.3: IBU-CyD complexation energy

CyD	Docking Score (S)	ΔE
α-CyD	-3.62	-3.04
β-CyD	-4.37	-10.28
γ -CyD	-3.67	-9.57

Thermodynamically, negative complexation energy is indicative of a spontaneous, exothermic process and the ability of the guest molecule to bind to the host [24,25]. Thus, Table 2.1.3 suggests the formation of IBU-CyD inclusion complexes for the CyDs evaluated. However, the values of ΔE obtained from the docking studies suggest slight differences between the complexation effects of γ-CyD and β-CyD on IBU. This is similar to the values obtained from the CE (Table 2.1.2). Wang and co workers [22] have made similar observations from a molecular docking study (using the Autodock Vina package) of IBU-CyD. While it is generally accepted that molecular docking is not enough for the complete theoretical elucidation of the CyD host-guest interaction, it readily provides a rapid insight into mechanism and geometry to allow a rational approach to drug development. For all the studies carried out, a correlation was observed between complexation ability of β-CyD and γ-CyD over α-CyD. This is possibly due to the small size of the α-CyD hydrophobic cavity and the consequent limited insertion of the IBU molecule as observed in Figure 2.1.2.

3.2 Study of LPV-CyD host guest interactions

One of the reasons for the derivatization of native CyD is the inability of native CyD to form inclusion complexes with high molecular weight guest molecules [26]. The addition of a third component to form CyD ternary systems with enhanced CE is a common practice in the development of CyD drug delivery systems [27]. Due to poor LPV uptake and low UV detection, a 50% hydroethanolic solution was used for the phase solubility studies. The phase solubility plots and calculated parameters are presented in Figures 2.1.3 to 2.1.5 and Tables 2.1.4 and 2.1.5 respectively. The plots generally show A_L type with an increase in the concentration of CyD resulting in an increase in the concentration of LPV (ethanol being

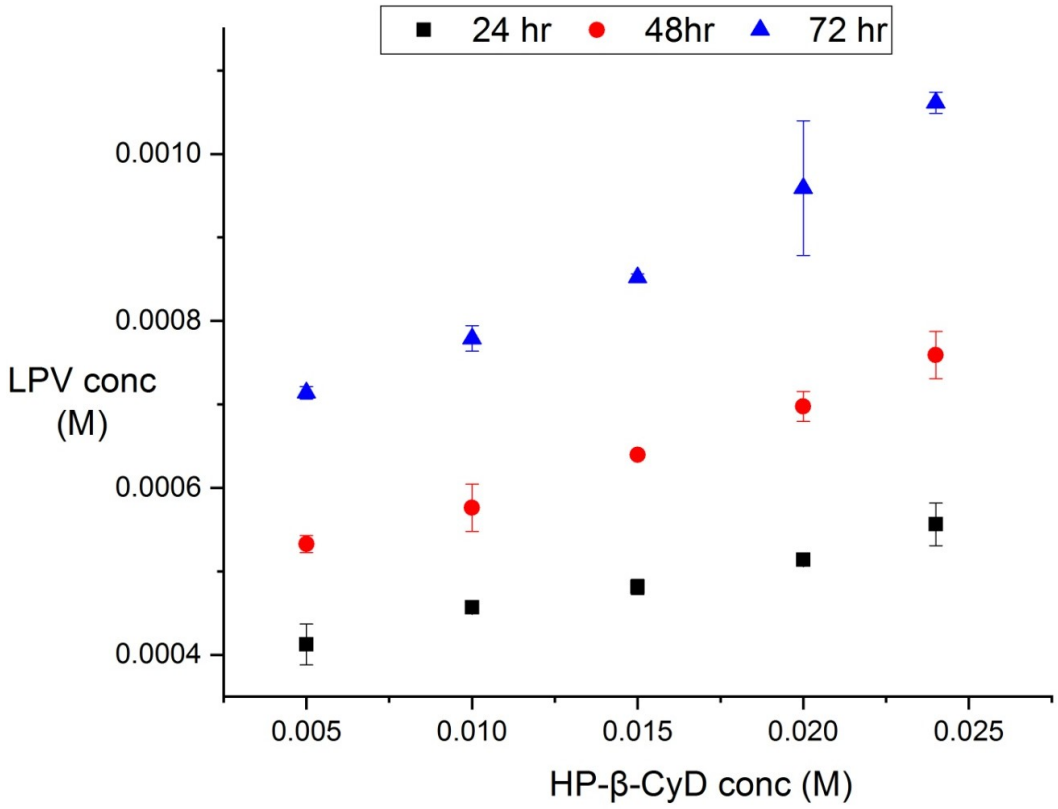


Figure 2.1.3: Phase solubility curves for LPV-HP-β-CyD complexes.

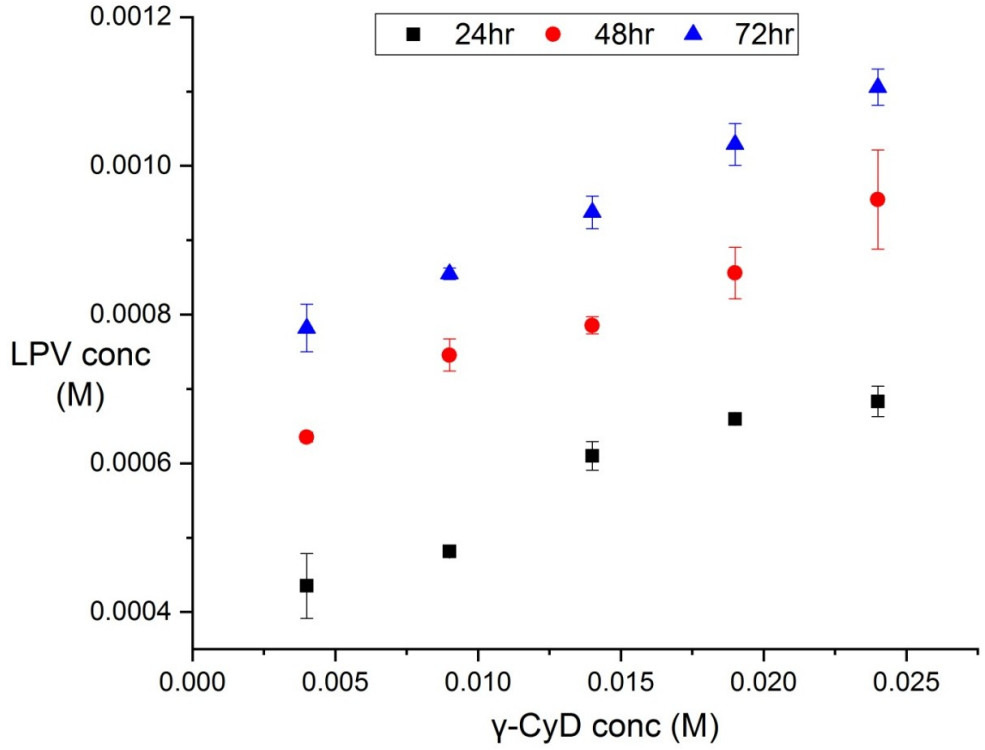


Figure 2.1.4: Phase solubility curves for γ-CyD complexes.

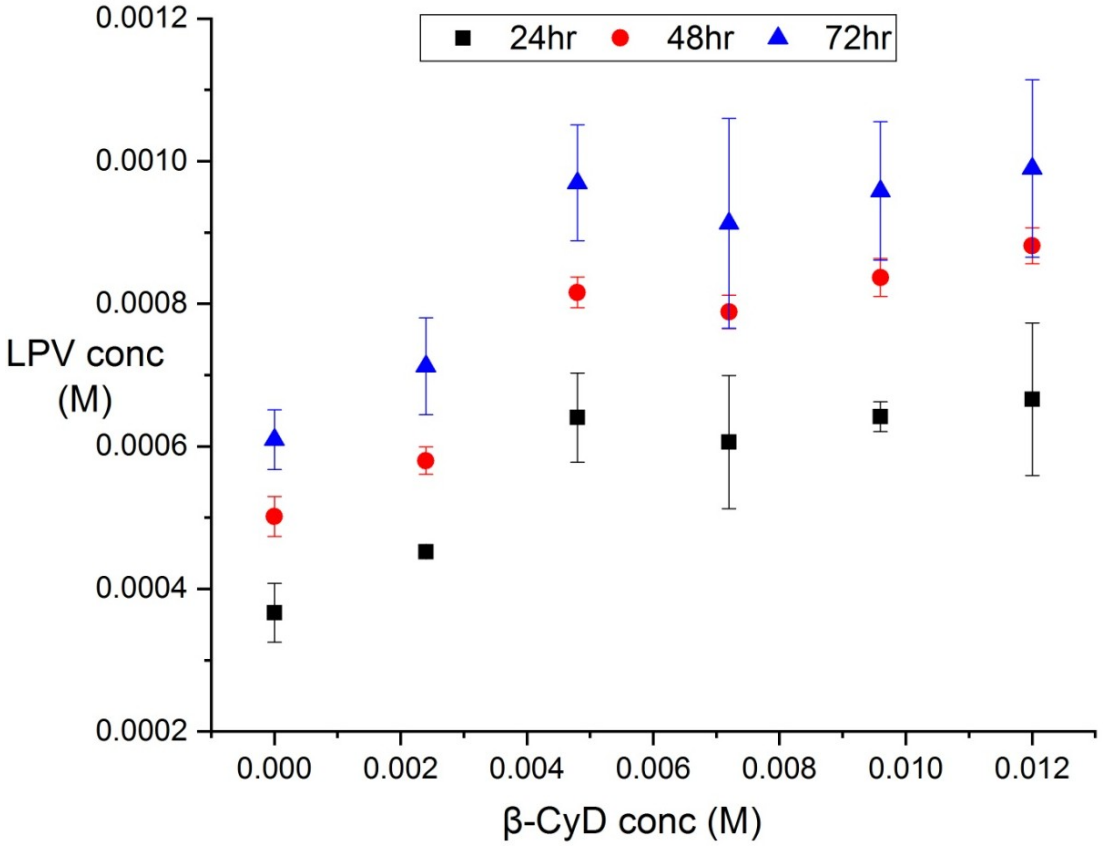


Figure 2.1.5: Phase solubility curves for β-CyD complexes.

Table 2.1.4: Phase-solubility study data of LPV and CyD

CyD	Slope			S_o (Intercept)			R^2		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
β-CyD	0.057	0.065	0.075	0.0003	0.0005	0.0006	0.955	0.923	0.943
HP-β-CyD	0.007	0.012	0.018	0.0004	0.0005	0.0006	0.986	0.993	0.981
γ-CyD	0.014	0.015	0.016	0.0004	0.0006	0.0007	0.940	0.980	0.999

Table 2.1.5: Phase-solubility studies of LPV and CyD continued.

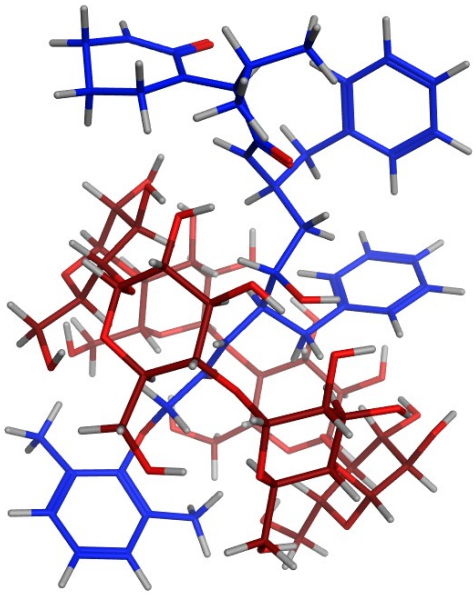
CyD	K1:1			CE			D:CyD		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
β-CyD	201.860	139.037	135.135	0.061	0.070	0.081	17.513	15.385	13.333
HP-β-CyD	18.131	24.087	30.723	0.007	0.012	0.018	138.889	84.034	55.249
γ -CyD	34.212	25.381	23.819	0.014	0.015	0.017	74.074	66.667	60.976

constant variable). While an increase in LPV solubilization with time can be observed from the plots, apparent stability constant shows decrease in binding interaction for both β-CyD and γ-CyD reiterating the potential for erroneous results due to the influence on S_{int} on the result. Complexation efficiency (CE) increased while D:CyD reduced with time indicative of enhanced equilibration. Despite the inclusion of ethanol in this system, complexation efficiency less than 0.1 for LPV whose dose is greater than 250mg suggests poor feasibility for oral delivery [4]. When compared to the results of the phase solubility studies, molecular docking studies also generally suggest poor LPV-CyD complexation. While the *ΔE* of β-CyD and γ-CyD (Table 2.1.6) is negative suggesting some form of molecular interaction, the values obtained are closer to those of observed in IBU complexation with α-CyD (Table 2.1.3). The positive *ΔE* values observed with α-CyD correlates with its inability to solubilise LPV in phase solubility studies. No correlation was found between in silico and experimental data for both γ-CyD and β-CyD. While CE suggests β-CyD > γ-CyD; ΔE suggests the opposite. However, both phase solubility and docking studies showed that LPV will more readily bind β-CyD to than HP-β-CyD. Also, it is generally accepted that CyD derivatization can increase cavity size by expanding the hydrophobic region, thus making CyD derivatives better suitable for complex formation [28], however, a phenomenon where CyD derivatives showed poorer complexation ability compared to their parent molecule has been reported in literature [29]. It has been suggested that steric and electronic factors of large substituent groups can negatively impact complexing ability by causing the self inclusion or occlusion of the cavity entrance; thereby preventing the entry of the guest molecule [28]. Also, the nature of the guest molecule and its physicochemical interaction with the CyD substituent groups

may influence the extent to which derivitization and/or degree of substitution increases or decreases complexation ability [30]. As observed from Table 2.1.6; complexation behaviour of CyD derivatives was dependent on the type and degree of substitution.

Table 2.1.6: LPV-CyD complexation energy

CyD	Docking Score (S)	ΔE
α-CyD	-4.982	26.99
β-CyD	-5.52	-2.02
γ -CyD	-6.09	-3.76
HP-β-CyD Model A	-6.805	12.83
HP-β-CyD Model B	-5.054	1.31
HP-β-CyD Model C	-6.699	14.23
HP-β-CyD Model D	-6.647	1.82
HP-β-CyD Model E	-6.851	16.84
SBE-β-CyD Model A	-6.691	14.41
SBE-β-CyD Model B	-7.127	4.69
SBE-β-CyD Model C	-6.689	8.39
SBE-β-CyD Model D	-7.265	14.83
SBE-β-CyD Model E	-6.282	14.05
HP-γ-CyD Model A	-7.390	6.00
HP-γ-CyD Model B	-6.889	-10.09
HP-γ-CyD Model C	-7.076	6.87
HP-γ-CyD Model D	-6. 326	7.23
HP-γ-CyD Model E	-6.485	-0.47



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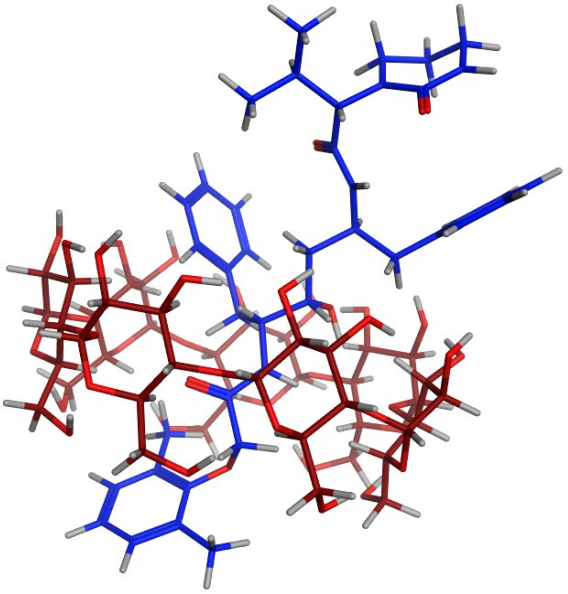

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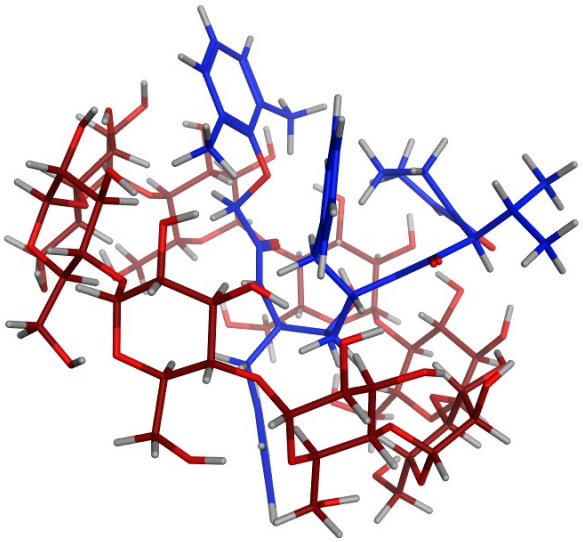

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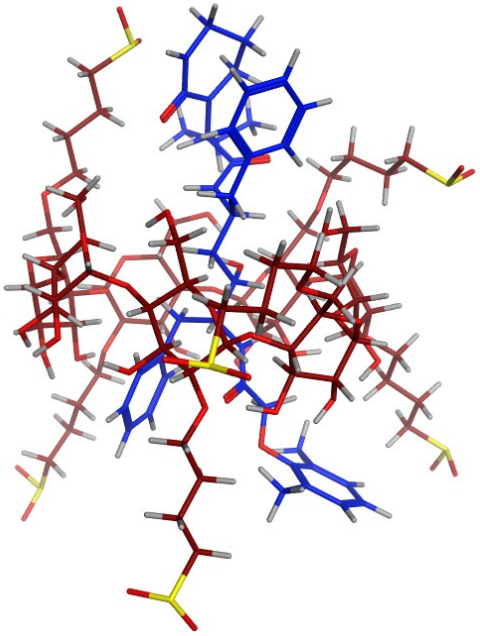

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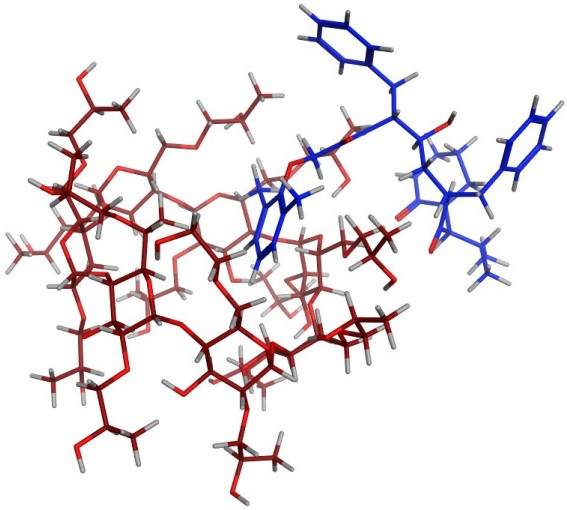

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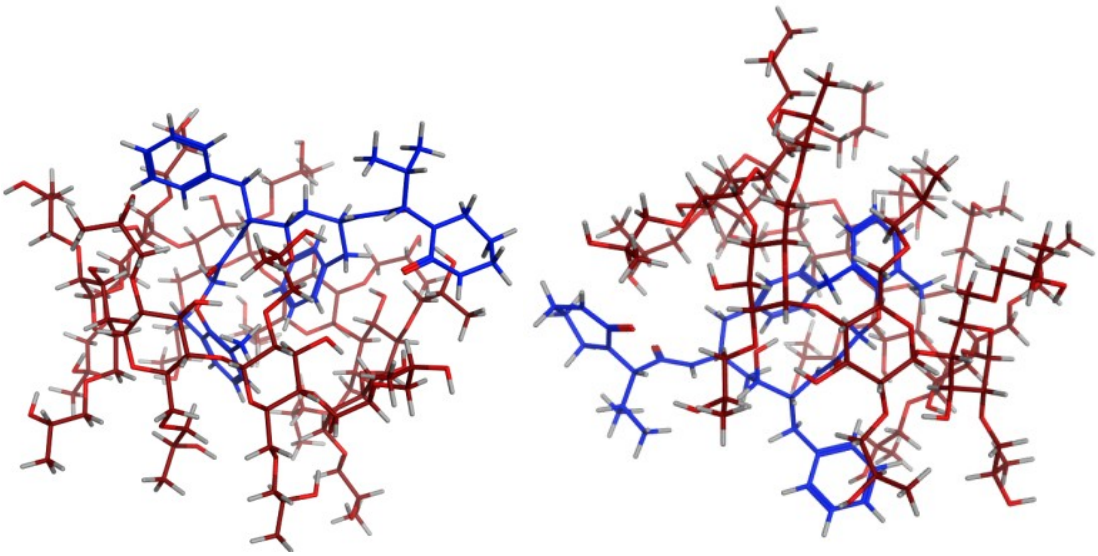

(f)

Figure 2.1.6 : Optimal models of LPV- CyD complexes obtained for molecular docking studies (a) LPV-α-CyD (b) LPV-β-CyD (c) LPV-γ-CyD (d) LPV-SBE-β-CyD (e) LPV-HP-β-CyD (f) LPV-HP-γ-CyD (two views)

4. Conclusion

The application of molecular docking for the study of LPV-CyD complex formation suggests that a HP-γ-CyD (DS≈16) may be suitable for the preparation of LPV-CyD complex. However, this type of HP-γ-CyD is not commercially available. Since the

synthesis/derivatization of γ-CyD with (2-hydroxyl)propyl can be partially controlled by varying the alkalinity (low and high alkalinity resulting in O2 and O6 substitution respectively)of the condensation reaction medium between CyD and propylene oxide, the successful synthesis and experimental analysis of the complexation ability of HP-γ-CyD (DS≈16) relative to γ-CyD and commercially available HP-γ-CyD (DS≈4.6) may further elucidate the utility of the docking protocol proposed in this study for CyD selection in the development of CyD drug delivery systems.

It is also important to note that beyond molecular docking, more complex in silico methods using quantum mechanics and molecular dynamics are able to provide deeper insight into CyD molecular interactions. However, the docking protocol on MOE offers a fast and user-friendly platform for drug formulation scientists who might not be particularly skilled in computational chemistry.

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Section 2

Preparation of ibuprofen/hydroxypropyl-γ-cyclodextrin inclusion complexes using supercritical CO_2 assisted spray drying

This section is adapted from a published article:

Oluwatomide Adeoye, Clarinda Costa, Teresa Casimiro, Ana Aguiar-Ricardo and Helena Cabral-Marques (2018) *Preparation of ibuprofen/hydroxypropyl-γ-cyclodextrin inclusion complexes using supercritical CO2 assisted spray drying.* The Journal of Supercritical Fluids. 133(1), 479-485

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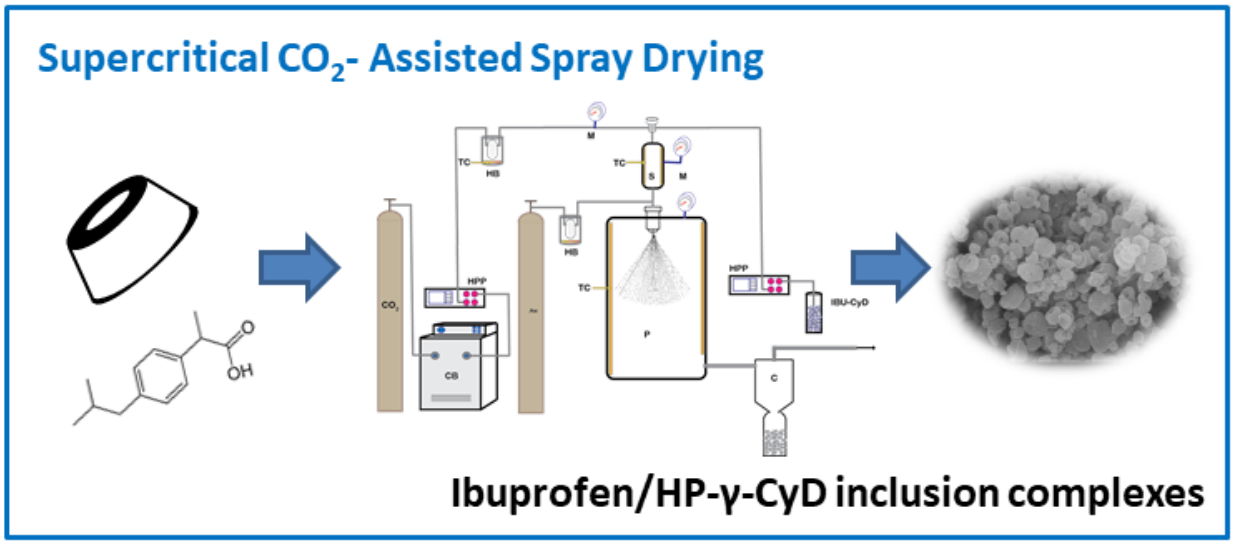
Abstract

Herein we report the formation of dry powder complexes of ibuprofen (IBU) and hydroxypropyl-γ-cyclodextrin (HP-γ-CyD) by supercritical fluid CO_2-assisted spray-drying (SASD). HP-γ-CyD alone, IBU and HP-γ-CyD, as well as the physical mixture were prepared and characterized using Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), X-ray diffraction (XRD), Ultra-violet spectroscopy (UV), ^{13}C cross-polarization magic angle spinning nuclear magnetic resonance (^{13}C CP/MAS NMR), Differential scanning calorimetry (DSC) and morphological studies. Results indicate the successful formation of amorphous inclusion complexes. SASD is a clean technology, suitable for processing thermolabile APIs, thus an interesting alternative to conventional spray drying and other methods of CyD solid complex formation.

Graphical Abstract



Highlights:

- Ibuprofen and hydroxypropyl-γ-cyclodextrin were successfully co-atomized by SASD
- Samples were characterized by ATR-FTIR, XRD, UV, ^{13}C CP/MAS NMR, SEM and DSC
- Results strongly suggest the formation of amorphous inclusion complexes
- SASD is a green alternative to conventional methods of CyD complex formation

1. Introduction

Cyclodextrins (CyDs) are cyclic oligosaccharides made up repeating glucopyranose units linked by α-(1,4) glycosidic bonds in a ring formation. CyDs importance in the pharmaceutical industry is largely due to their ability for host-guest molecular interactions (inclusion and non-inclusion complexes) with various active pharmaceutical ingredients (APIs). This enables CyDs to modify drug delivery properties such as aqueous solubility, physicochemical and physiological stability; and drug release/targeting properties *in vivo*. The formation of inclusion complexes is mediated by apolar attraction of lipophilic drug molecules to CyDs' interior cavities lined with ethereal carbons, while the orientation of their hydroxyl groups makes their exterior surfaces hydrophilic [1,2].

Several methods have been reported for the preparation of solid drug-CyD complexes. Examples include spray-drying, lyophilization, kneading, milling/co-grinding, co-evaporation, co-precipitation, microwave irradiation, etc [3-5]. The choice of preparation method has been reported by several authors to affect the physicochemical properties and physiologic performances of CyD based drug delivery systems [5-7]. Supercritical fluids (SCFs) technology has emerged as a very important method for various pharmaceutical processes, including CyD complex formation [8]. It utilizes dense gases with high compressibility, diffusivity and evaporation rate, which are tuneable under varying conditions of temperature and/or pressure, to facilitate the efficient manipulation of solvent effect on APIs and drug carriers. Also, SCF technology is greener, sustainable, low cost, non-toxic; and generally reduces the complexity of pharmaceutical unit operations during particle preparation or engineering [8,9]. Many types of SCFs processes with specific merits and demerits have been advanced for pharmaceutical processes. Examples include: Rapid Expansion of Supercritical Solution (RESS), Rapid Expansion of a Supercritical Solution into a Liquid Solvent (RESOLV), Gaseous Antisolvent (GAS), Particles by Compressed Antisolvent (PCA), Supercritical Antisolvent (SAS), Solution Enhanced Dispersion by Supercritical Fluids *(SEDS)*, Supercritical Fluid-Assisted Atomization/Spray Drying (SAA/SASD), etc. [8].

Supercritical Assisted Atomization/Spray Drying has been described as one of the most effective micronization techniques for the production of spherical and amorphous micro and sub-microparticles [10]. First introduced by Reverchon and coworkers [11], the process uses CO_2 as a co-solute, that is, the supercritical CO_2 is solubilised into the liquid solution

containing the drug-carrier system. This solution is then sprayed to a precipitator, at near atmospheric pressure, via a nozzle. The low surface tension (near zero) and viscosity of this expanded solution improve the atomization process by facilitating the production of droplets that are rapidly dried, preventing the organization of solute molecules into ordinate forms representative of crystals [10,12]. More recently, Cai and co-workers[13] have shown that the introduction of a hydrodynamic cavitation mixer to SAA can enhance mass transfer in order to reduce processing time, and make the system more conducive for thermolabile materials such as biopharmaceuticals [13,14].

Since the turn of the millennium, several research groups, have prepared solid drug CyD complexes using different types of SCFs technology [6,15-21]. The micronizing effect of SASD on CyD has been previously studied by Reverchon and Antonacci [20]. However, to the best of our knowledge, the description of SASD's utility in the preparation of solid drug-CyD complexes has not been reported in literature. Compared to conventional spray drying, SASD offers several advantages. Firstly, SASD allows for narrow and controlled particle size distribution required in the development of pulmonary and nanoparticulate drug delivery systems [22]. By acting as an efficient pneumatic agent, fast elimination of CO_2 from the primary droplets (decompressive atomization) during atomization leads to the production of smaller secondary droplets and particles [23]. Secondly, it is preferable for thermo-labile drugs since it utilizes lower operation temperature and eliminates the drying step required to reduce residual solvents present in spray dried particles to acceptable limits. Thirdly, when desired, the solubilisation effect of $scCO_2$ allows for effective cross-linking and/or intermolecular interactions between APIs and drug carrier systems in order to modify their physicochemical and physiologic properties [24-26]. Thus, when used to prepare solid drug-CyD complexes, $scCO_2$ can act as a co-solvent/co-solute in a CyD ternary system with huge possibilities for enhancing the formation of inclusion complexes.

In this study, we report the formation of solid complexes of ibuprofen (IBU) and hydroxypropyl-γ-cyclodextrin (HP-γ-CyD) by SASD. SASD of HP-γ-CyD alone and IBU/HP-γ-CyD, as well as the physical mixture were prepared and characterized using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), X-ray diffraction (XRD), ultra-violet spectroscopy (UV), ^{13}C cross-polarization magic angle spinning nuclear magnetic resonance (^{13}C CP/MAS NMR), differential scanning calorimetry (DSC) and morphological studies.

2. Experimental

2.1. Materials

(±)-Ibuprofen 20/35 (racemic α-methyl-4-[2-methylpropyl] benzene acetic acid, IBU) and CAVASOL® (hydroxypropyl-gamma-cyclodextrin, HP-γ-CyD) were kind gifts from Laboratórios Medinfar (Portugal) and Wacker Chemie AG (Germany), respectively. Ethanol (absolute anhydrous, 99.9% purity) was purchased from Carlo Erba Reagents. The water used in this study was purified with a Milli-Q water purification system (Water Max W1, Diwer Technologies). Industrial carbon dioxide (purity ≥ 99.93%) was purchased from Air Liquide. All compounds were used as received without further purification.

2.2. Methods

2.2.1. Preparation of IBU/HP-γ-CyD inclusion complex by SASD and Physical mixture.

Solid complexes of IBU and HP-γ-CyD were prepared by SASD. Based on previous studies [7,27-29], a molar ratio of 1:1 was used. Both compounds were dispersed in 60 mL of a hydroalcoholic solution 1:1 (v/v), vortexed and then sonicated in an ultrasound bath (Sonorex RK 100H) for 15 min to facilitate complete dissolution. The resulting complex solution was then filtered (80 μm filter). Two high-pressure pumps were used to deliver the complex solution (HPLC pump 305 Gilson) and the $scCO_2$ (HPLC pump K-501, Knauer) into a high-pressure static mixer saturator (3/16 model 37-03-075, Kenics Chemineer, 4.8 mm diameter, 191 mm length and 27 helical mixing elements) to facilitate the near equilibrium mixing of the $scCO_2$ and the complex solution. The CO_2 was first liquefied in a cryogenic bath, heated in an oil bath before entering the static mixer at a flow rate of 25 mL/min while the complex solution was fed into the static mixer at a flow rate of 2 mL/min. The pressure (12.8 MPa) was measured by a Setra pressure transducer and the temperature of the mixture, at the static mixer (65 °C) was controlled by a Shinko FCS-13A temperature controller. All operation parameters were selected based on previously optimized conditions in our laboratory and drug specific requirements found in literature. The mixture was then atomized through a nozzle with an internal diameter of 150 μm into the precipitator (an aluminium vessel that operates at near-atmospheric conditions). At the same time, a flow of previously heated compressed air (T = 120 °C) entered the precipitator to evaporate the liquid solvent. The temperature measured at the exit of the precipitation chamber was around 70 °C. The formed particles were then separated from the Air-CO_2-solvent flow by the high

efficiency Büchi cyclone. A solution of HP-γ-CyD (without IBU) was also subjected to SASD using the same conditions. All powders were then collected and stored in a screw capped glass bottle prior analysis.

For the physical mixture (PM), regarded as control, IBU and HP-γ-CyD (1:1) were weighed into in flask and thoroughly mixed on a vortex, for 10 min. The particles obtained were also stored in a screw capped glass bottle prior analysis.

2.2.2. Morphological Characterization

The morphology of the atomized particles as well of the physical mixture were observed by scanning electron microscopy (SEM) using a Hitachi equipment (model S-2400), with an accelerating voltage set to 20 kV. All samples were mounted on aluminium stubs using carbon tape and gold coated before analysis. Particle size distribution was determined using an optical particle analyser system (Morphologi G3 from Malvern Instruments Ltd., Malvern, UK). Atomized particles were characterized for mean particle size and particle size distribution by considering more than 30,000 particles. This characterization was performed in terms of the volume mean diameter (D_v) and the relative width of the distribution (span). The span is calculated using three measures, $D_{v,10}$, $D_{v,50}$, and $D_{v,90}$ (particle volume diameter corresponding to 10%, 50%, and 90% of the population, respectively) by the following equation:

$$Span = \frac{D_{v,90} - D_{v,10}}{D_{v,50}} \qquad \text{Eq. 1}$$

2.2.3. Differential Scanning Calorimetry (DSC)

The thermal behaviour of all samples was analysed on a DSC TA Q1000 (TA Instruments, New Castle, Delaware, USA). Each powder (3.5 mg) was placed in a pin-holed DSC aluminium pans and heated at 10 °C/min from 40 up to 300 °C under continuous dry nitrogen purge (50 mL/min). Data was analysed and processed using the TA Universal Analysis 2000 Software (TA Instruments, New Castle, Delaware, USA).

2.2.4. X-ray diffractometry (XRD)

Powder X-ray diffraction analysis was performed in a RIGAKU X-ray diffractometer (model Miniflex II) with automatic data acquisition (Peak search for Windows v. 6.0 Rigaku). Samples placed in a holder were analysed using CuKα radiation (30 kV/15 mA) and

the diffraction patterns were collected with a 2θ angle ranging between 5° and 55° and a scan rate of 0.02°/min.

2.2.5. Attenuated Total Reflectance-Fourier Transform-Infrared spectroscopy (AT-FTIR)

AT-FTIR spectra were obtained on a Perkin-Elmer Spectrum Two spectrometer (PerkinElmer, USA). Suitable amount of each powder was applied on the diamond plate surface, completely covering the surface of the prism. The spectrum was recorded (16 scans for each spectrum) from 450 to 4000 cm^{-1}.

2.2.6. Ultraviolet spectroscopy (UV)

A Perkin-Elmer 400 UV-spectrophotometer was used to verify the presence of IBU in the SAA prepared complex. Briefly, 0.0128 g of each powder was weighed, solubilised in 10 mL ethanol and vortexed for 2 min to ensure complete dissolution. The absorbance of the solution was scanned from 400 to 200 nm and compared with that of the free drug.

2.2.7. ^{13}C Cross-Polarization Magic Angle Spinning CP/MAS Nuclear magnetic resonance (NMR)

The solid-state ^{13}C CP/MAS NMR measurements of all samples were recorded on a Bruker NMR (Avance III 300MHz WB) spectrometer using a 4mm MAS multinuclear probe. Each spectrum was recorded with an angular velocity of 5 kHz and 3500 accumulations. The acquisition parameters were AQ (0,05 s); DW (10 us); DE (20 us), D1 (2 s).

2.2.8. Assay of Drug Content

The total of amount of drug (IBU) present in each powder was determined by the UV spectrophotometry. 5 mg each of IBU/HP-γ-CyD SASD complex and physical mixtures was dissolved in 10 mL of hydroethanolic solution (1:1 v/v) and subjected to ultrasonication for 25 min to allow complete dissolution of the entrapped active compound. After this procedure, the solutions were centrifuged at 2500 rpm for 30 min to remove any undissolved particles. 3 mL of the supernatant was collected, filtered with a 0.45 μm membrane filter (PTFE) and analysed by UV. The percentage of drug content was calculated according to the equation:

$$Drug\ Content\ \% = \frac{IBU\ content}{Initial\ IBU\ amount} \times 100 \qquad \text{Eq.2}$$

where "IBU content" is the amount of drug present in the solid complex and physical mixture, and "initial IBU amount" indicates the IBU amount initially used to prepare the samples.

3. Results and discussion

3.1 Morphology

The morphology of the samples was assessed by scanning electron microscopy. Figure 2.2.1 shows SEM micrographs of the physical mixture, and of the particles obtained from processing CyD alone and IBU /HP-γ-CyD by SASD. The physical mixture is formed by very large discrete particles up to 200 μm (Figure. 2.2.1a). The particles obtained by atomization are very homogeneous. The HP-γ-CyD particles (Figure 2.2.1b) have quite irregular surfaces while the particles containing the complexes (Figure 2.2.1c) have smother surfaces.

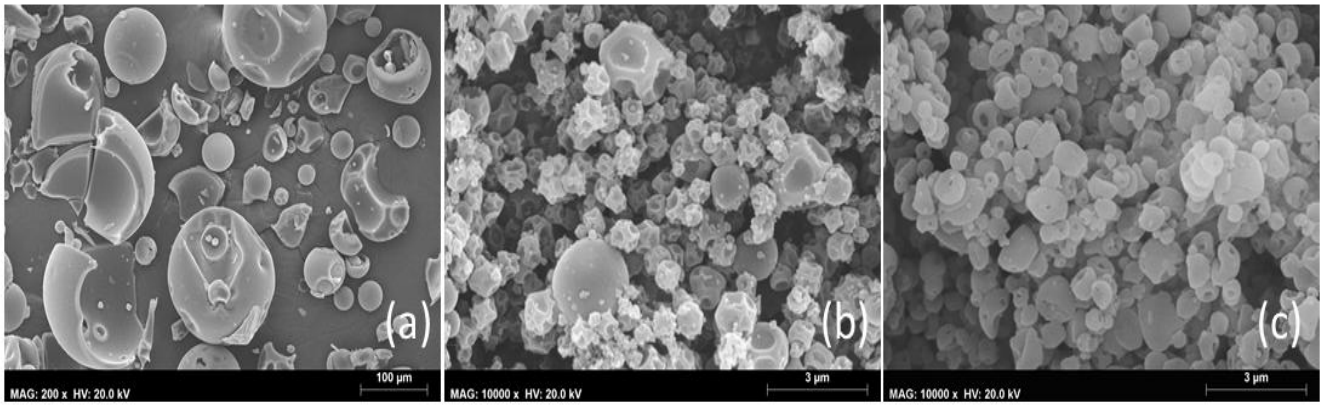


Figure 2.2.1. Scanning electron microscopy micrograph images of the physical mixture-200x (a) and of the SASD processed HP-γ-CyD -10000x (b) and Ibuprofen+ HP-γ-CyD -10000x (c).

Particle size and particle size distribution for SASD processed HP-γ-CyD and IBU/HP-γ-CyD samples are shown in Table 2.2.1 and Figure 2.2.2 Both samples show very homogenous particle size distribution as it was proved by their narrow span values. In addition, the IBU/HP-γ-CyD sample has shown a slightly lower particle mean diameter compared to the processed CyD.

Table 2.2.1: Properties of SASD processed HP-γ-CyD and IBU/HP-γ-CyD.

Sample	D_n (μm)	$D_{v,10}$	$D_{v,50}$	$D_{v,90}$	Span
HP-γ-CyD	2.99	7.31	27.20	57.97	1.86
IBU/HP-γ-CyD	2.28	11.54	36.54	58.30	1.27

$D_{n,50}$–Particle number mean diameter; $D_{v,x}$- Particle volumetric diameter of x% cumulative distribution; Span determined according to Eq. (1).

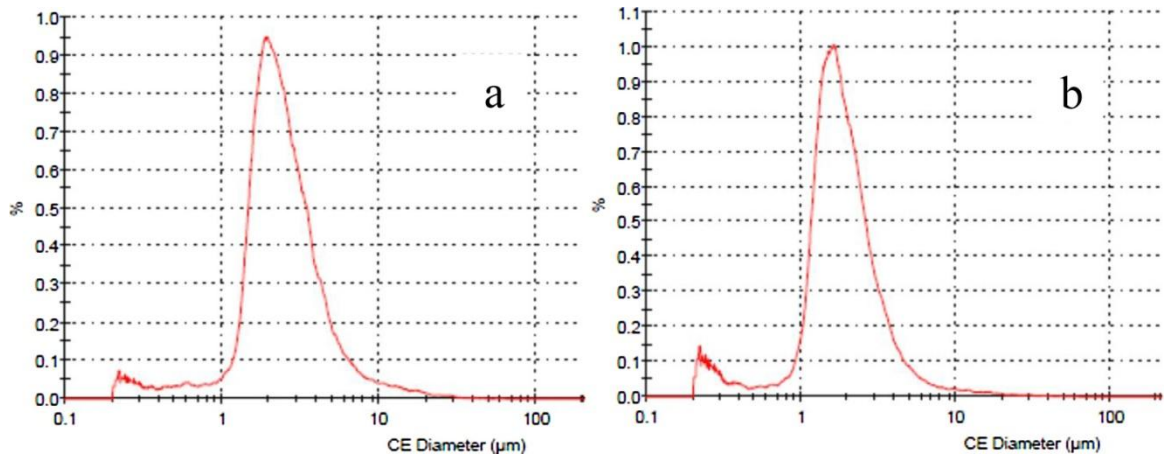


Figure 2.2.2: Particle size distribution (number distribution) from Morphologi G3 report, for: a) HP-γ-CyD and b) IBU/HP-γ-CyD .

3.2 Differential scanning calorimetry analysis

Thermal analysis is considered an important tool for assessing changes in thermal behaviour due to solid state interactions between drug and CyDs. Typically, the melting endothermic peaks of the formed complex are compared with those of single components and observed changes like reduction, broadening and/or shifts of these peaks are considered indicative of the formation of complete or partial inclusion complexes [30,31]. Figure 2.2.3 presents the DSC thermograms of pure IBU, HP-γ-CyD and IBU/HP-γ-CyD complex prepared by SASD, and their physical mixture. The DSC thermograms for IBU shows a sharp melting endotherm at approximately 77 ^oC indicating the crystalline nature of the drug while that of HP-γ-CyD displayed the typical dehydration induced broad endothermal peak of CyDs which is usually observed below 130^oC [30,32]. For the complex prepared by SASD, the complete disappearance of the IBU melting peak suggests the complete inclusion of the drug into the

cavity of HP-γ-CyD [33-35]. While it has been suggested that this could be related to the formation of amorphous systems and/or inclusion complexes [31], these other phenomena are typically characterised by the presence of a reduced melting peaks (not complete disappearance) [36-38]. For the physical mixture, while the negative shift in the melting endothermal peak of crystalline IBU and its reduced intensity suggests some form of solid state interaction between IBU and HP-γ-CyD. The residual presence of IBU's melting endothermal peak suggests the conversion of IBU into amorphous form and/or partial or incomplete inclusion characterized by the presence of free IBU molecules outside the cavity of HP-γ-CyD [31,39] Also, the slight reduction in the intensity and the negative shift in dehydration induced endothermal peak of HP-γ-CyD also suggests SASD induced formation of inclusion complex [30,32].

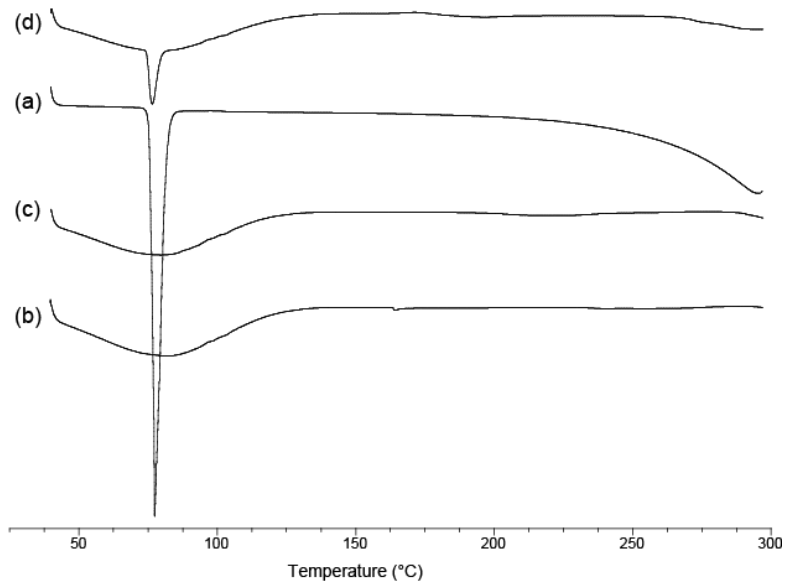


Figure 2.2.3: DSC thermograms for (a) IBU, (b) HP-γ-CyD**,** (c) IBU/HP-γ-CyD complex and (d) IBU/HP-γ-CyD physical mixture.

3.3 X-ray powder diffraction analysis

Figure 2.2.4 presents the X-ray diffraction patterns of pure IBU, HP-γ-CyD and IBU/HP-γ-CyD complex prepared by SASD, and their physical mixture. Changes in the XRD patterns of drug such as shifts or complete disappearance of crystalline peaks, reduction in peak intensity, appearance of new diffraction peaks or completely diffused patterns generally

suggests complete drug amorphization and/or complex formation [31,40]. As shown on Figure 2.2.4, the XRD patterns of IBU (Figure 2.2.4a) displayed sharp and intense peaks indicative of its crystalline nature, while that of HP-γ-CyD (Figure 2.2.4b) showed 2 broad halos, thus confirming its amorphous nature. The diffractogram of the SASD prepared complex retained the XRD pattern of HP-γ-CyD with its two broad halos at 11^o and 18^o (Figures 2.2.4b and 2.2.4c). While XRD analysis alone cannot provide a conclusive evidence of complex formation, if interpreted with the DSC data, the observed diffused pattern of SASD particles and the absence of the drug crystalline peaks enhances the hypothesis of inclusion complex formation over drug amorphization [6,41]. On the other hand, the diffractogram of the physical mixture (Figure 2.2.4d) retained the sharp crystalline peaks of ibuprofen (Figure 2.2.4a) indicating the presence of the drug as a crystalline material in the binary mixture. The decrease in peak intensity of IBU can be ascribed to the amorphous nature of the HP-γ-CyD [34]. While several authors have interpreted the presence of drug

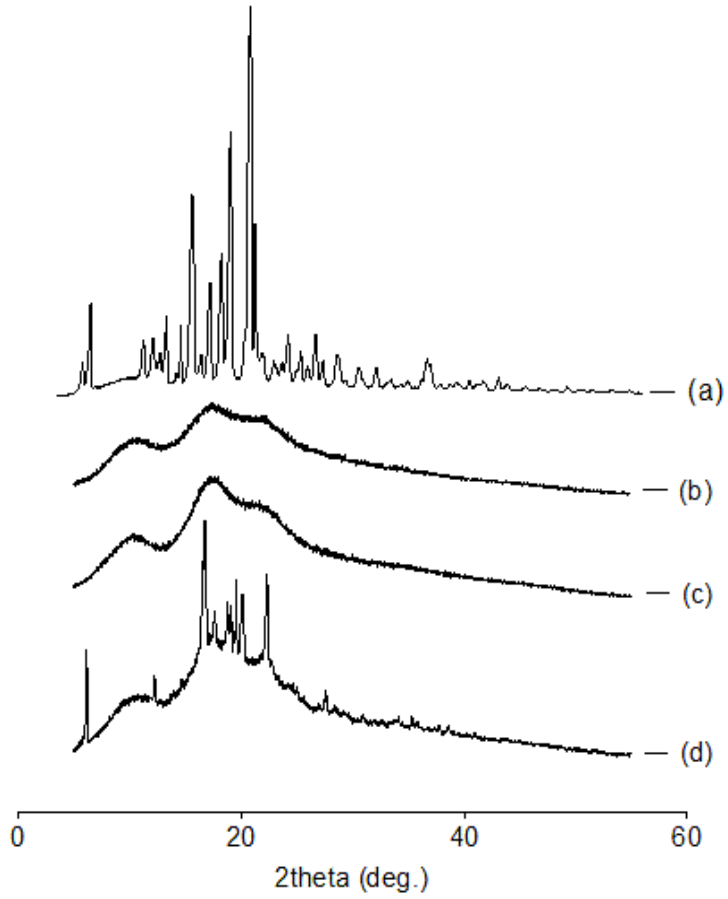


Figure 2.2.4. X-ray diffractograms (a) IBU (b) HP-γ-CyD (c) IBU/HP-γ-CyD complex (d) IBU/HP-γ-CyD physical mixture.

Figure peaks in the diffractogram of physical mixtures as the absence of complex formation [34,42], it could also be due to drug amorphization and/or incomplete inclusion complex formation [34,43].

3.4 Attenuated Total Reflectance-Fourier Transform-Infrared spectroscopy

Spectroscopic changes in vibrational modes of the IBU and SASD treated HP-γ-CyD are compared with those of the complex and physical mixture. The broad prominent stretching vibrations of HP-γ-CyD observed between 3000-3600 cm^{-1} (Figure 2.2.5) is typical of the O-H bonds of the primary and secondary hydroxyl groups of CyD; while vibration at 2932 cm^{-1} represents their CH and CH_2 groups. Typically, this region rarely produces evidence of inclusion complex formation since the higher weight content of CyD (in equimolar CyD-drug systems) results in significant overlapping and masking of these groups in the drug [31,38]. The symmetrical bending band of water present in CyD cavities which is normally observed between 1650 and 1630 cm^{-1} [44,45] was observed (1645.31 cm^{-1}) in all samples. The weakening of IBU's inter-atomic bonds in a different environment such as the cavity of HP-γ-CyD normally results in the restriction of its stretching vibrational mode; characterized by disappearance, broadening and/or shifts in the wave number and peak intensity of both IBU and HP-γ-CyD [31,46].

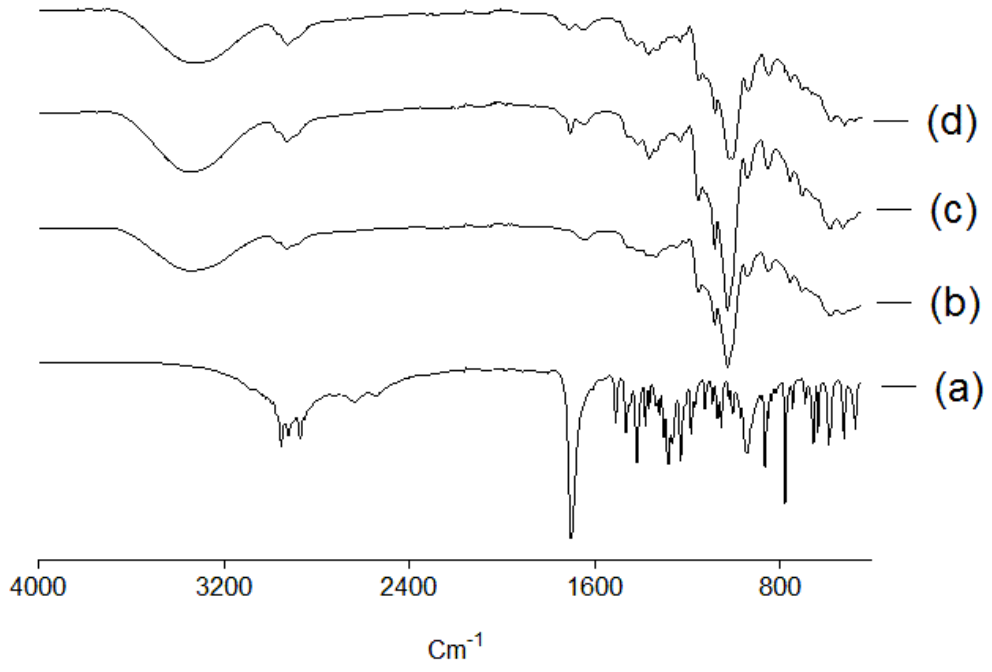


Figure 2.2.5: ATR-FTIR spectra for (a) IBU (b) HP-γ-CyD (c) IBU/HP-γ-CyD complex (d) IBU/HP-γ-CyD physical mixture.

As shown in Figure 2.2.5, IBU displays a strong carbonyl group stretching band at 1708.68 cm^{-1} [47,48]. While this band was completely absent in the HP-γ-CyD, its intensity was reduced in the spectrum of the complex and physical mixture. This is in agreement with our previous report [7] indicating the breakdown in the intermolecular hydrogen bonds of the crystals associated with IBU dimmers and the establishment of weaker forces after complex formation with CyD. Also, broadening or shifts in the wave number of IBU's carbonyl band observed in the physical mixture has been previously ascribed to incomplete inclusion in CyD cavity [7]. For the SASD formed complex, the wave number remained the same suggesting complete inclusion of IBU in the cavity of HP-γ-CyD. Other IBU vibrational stretching bands that were not as strong as that of the carbonyl group (1708.68 cm^{-1}) such as those between 2868 and 2957 cm^{-1}, 521.53 and 1481.61 cm^{-1} were also reduced in intensity and/or broadened by the formation of inclusion complexes.

3.5 Ultraviolet spectroscopy

The formation of drug inclusion complexes can cause significant changes to the UV absorption spectrum of the drug molecule. These changes can occur in response to the replacement of drug's solvation shell by CyD molecules and the position of the drug chromophore within the CyD cavity. Bathochromic or hypsochromic shift of the drug's absorption maximum and/or increase or decrease in its intensity have been suggested to be consequent of inclusion complex formation [48]. However, some studies have reported the absence of either bathochromic or hypsochromic after inclusion complex formation [45,48,49]. As observed in Figure 2.2.6a, the absorbance of pure IBU and the SASD prepared complex observed between 280 and 200nm were perfectly superimposed with the sharpest peak at about, approximately, 265nm. In the absence of spectra changes, the UV analysis only served to confirm the presence of IBU in the SASD prepared complex. The drug entrapped of both the SASD prepared complex and physical mixtures was also analysed by UV. Figure 2.2.6b shows the percentage of IBU present in the both SASD prepared complex and physical mixture. For the physical mixture, 91% of IBU was present, while the SASD prepared complex only allowed for 49% of the drug. Several authors have reported the influence of experimental and formulation parameters on final drug content of CyD complexes prepared by supercritical fluids[50,51]. It is possible that the optimisation of these parameters can lead to higher drug content in the SASD prepared complexes.

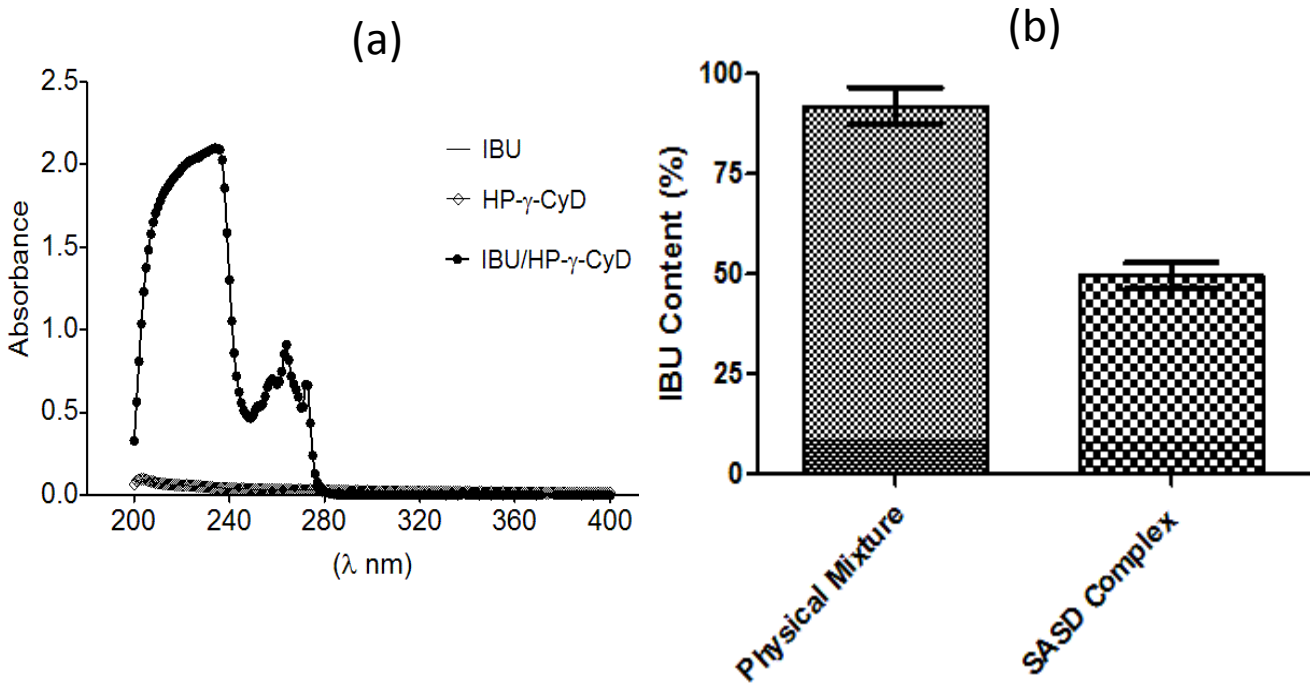


Figure 2.2.6. (a) UV Absorbance spectra for IBU, HP-γ-CyD and IBU/HP-γ-CyD complex; (b) Percentage drug content in the SASD prepared complex and physical mixture.

3.6 ^{13}C Cross-Polarization Magic Angle Spinning CP/MAS Nuclear magnetic resonance

Figure 2.2.7 shows the ^{13}C CP/MAS spectra and the assignment of carbon resonance for the evaluated samples. The HP-γ-CyD spectrum and ^{13}C signals are similar to those earlier reported for glucopyranose units of HP-β-CyD (the only structural difference being the number of units) while IBU spectra shows well-resolved ^{13}C signals with sharp resonances of a crystalline system similar to those previously reported by Skorupska and co-workers [52]. The disappearance of the ^{13}C resonance signals of IBU in the spectra of SASD prepared complex suggests IBU's intermolecular interaction with HP-γ-CyD and complete inclusion its cavity [53]. On the other hand, the reduced intensity of the ^{13}C resonance signals of IBU in the physical mixture indicates incomplete inclusion even though there was intermolecular interaction. This result is consistent with those of DSC, XRD and FTIR.

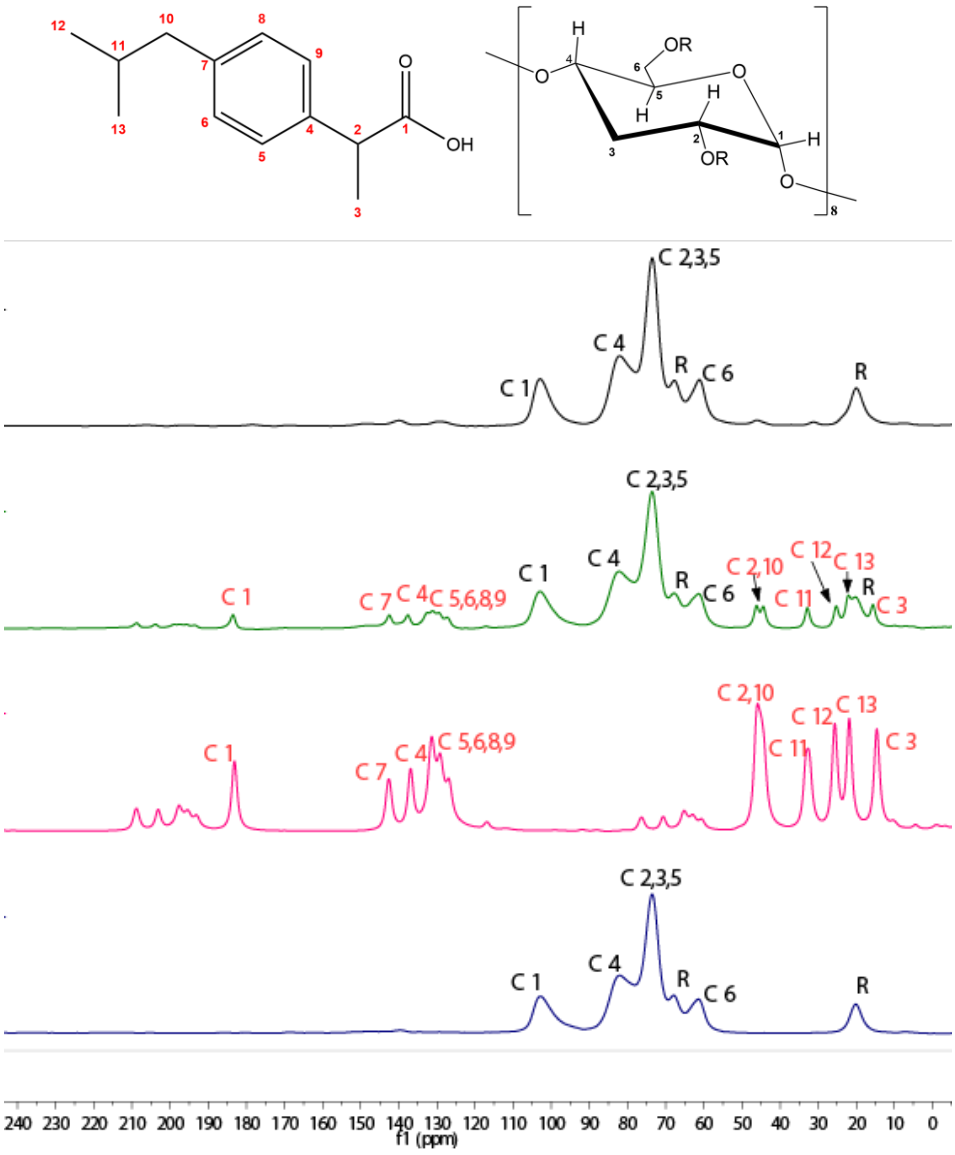


Figure 2.2.7 ^{13}C CP/MAS NMR spectra for IBU (pink), HP-γ-CyD (blue), IBU/HP-γ-CyD complex (black) and IBU/HP-γ-CyD physical mixture (green).

4. Conclusions

This study highlights the promising approach of supercritical assisted spray drying in the preparation of cyclodextrin inclusion complexes. The use of several analytical techniques such as DSC, XRD, AT- FTIR, UV, ^{13}C CP/MAS-NMR; and the combined evaluation of their results indicates the formation of amorphous inclusion complexes. Morphological analysis further confirms the utility of SASD in the production of solid complexes with homogeneous particle size. Considering that SASD is non-toxic, cheap and more suitable for

thermolabile APIs, it seems an interesting alternative to conventional spray drying and other methods of CyD solid complex formation. Also, the advantages of efficient particle size control inherent in decompressive atomization of SASD is particularly useful for the development of pulmonary and nanoparticulate drug delivery systems.

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CHAPTER 3

Cyclodextrin solubilization and complexation of antiretroviral drug lopinavir: *In silico* prediction; effects of derivatization, molar ratio and preparation method

This chapter is adapted from a published article:

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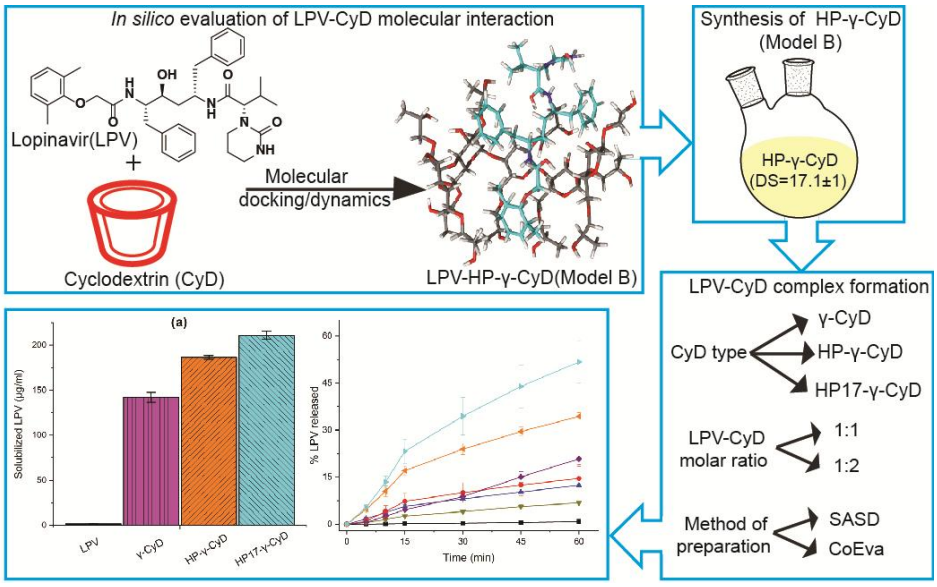
Abstract

Lopinavir (LPV) is currently used in combination with ritonavir for the clinical management of HIV infections due to its limited oral bioavailability. Herein, we report the application of an *in silico* method to study cyclodextrin (CyD) host-guest molecular interaction with LPV for the rational selection of the best CyD for developing a CyD based LPV delivery system. The predicted CyD, a (2-hydroxy)propyl-gamma derivative with high degree of substitution (HP17-γ-CyD) was synthesized and comparatively evaluated with γ-CyD and the commercially available HP-γ-CyD. All complexes were prepared by supercritical assisted spray drying (SASD) and co-evaporation (CoEva) at molar ratios (1:1 and 1:2); and afterwards fully characterized. Results indicate a higher LPV amorphization and solubilization ability of HP17-γ-CyD. The SASD processing technology also enhanced LPV solubilization and release from complexes. The application of *in silico* methodologies is a feasible approach for the rational and/or deductive development of CyD drug delivery systems.

Graphical Abstract



Highlights

- The best CyD for LPV complexation was predicted by *in silico* studies.
- The predicted CyD was successfully synthesized (HP17-γ-CyD).
- LPV complexes were prepared by two methods i.e. SASD and CoEva.
- HP17-γ-CyD facilitated higher LPV amorphization and solubilization.
- SASD processing technique enhanced LPV complexation and solubilization.

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1. Introduction

Lopinavir (LPV) (Fig. 3.1.1a), a potent inhibitor of HIV-1 protease is currently used in the clinical management of Human Immunodeficiency Virus (HIV) infections [1,2]. LPV belongs to the Class 4 of the Biopharmaceutical Classification System (BSC) [3,4], that is; it has a low oral bioavailability due to the combined effect of low aqueous solubility, P-glycoprotein (P-gp) efflux transport/low gastric permeability; and cytochrome P450 (CYP450 3A) metabolism [5-7]. Nowadays, LPV is clinically available as a co-formulation with suboptimal doses of ritonavir (RTV) which enhances oral bioavailability by inhibiting LPV's metabolism [2,5,8]. Adverse side effects of RTV such as; lipid elevation, perioral paraesthesia, glucose and gastrointestinal intolerance have fostered the development of a RTV free LPV formulation [2,9-11]. Several drug delivery systems based on poly(lactide-co-glycolide) [12], pullulan [2], poly-ε-caprolactone [13], lipid systems[14] etc. have been recently developed and evaluated for their ability to enhance the pharmacokinetic profile of LPV without the use of RTV.

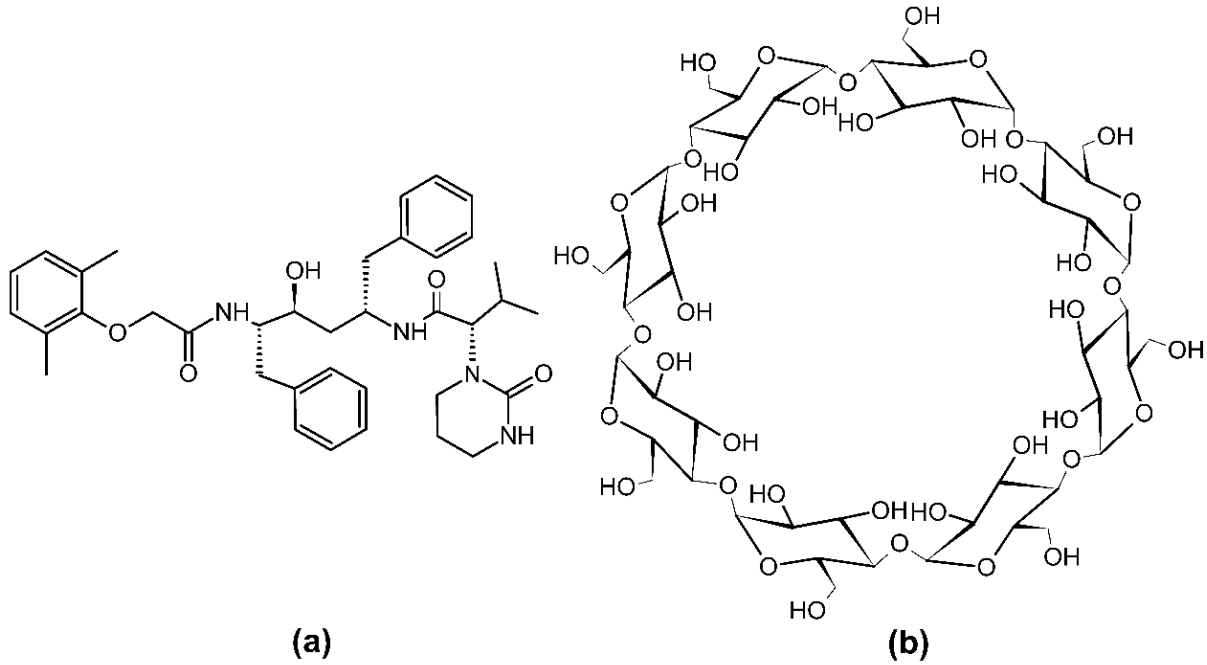


Figure 3.1.1. Chemical structure of (a) Lopinavir and (b) γ-Cyclodextrin.

Cyclodextrin (CyD) (Fig. 3.1.1b) is a class of cyclic oligosaccharides of α(1→4)glucopyranosides, and the most common, commercially available ones have 6, 7 or 8 (α, β, γ) units. Structurally, CyDs have a hydrophilic outer surface and a considerably less

hydrophilic central cavity that allows it to form complexes with several molecules, thereby modifying their physicochemical properties. The importance of CyD as drug carriers in the pharmaceutical industry is underlined by their ability to: (i) enhance drug aqueous solubility, bioavailability and physicochemical/physiological stability; (ii) modulate, target and/or control *in-vivo* drug release and pharmcodynamic activity, and (iii) reduce adverse drug reactions [15-17]. The ability of CyD to act as an efficient drug carrier depends on the type of CyD used in formulation development. Thus, several CyD derivatives have been synthesized with varying abilities for drug complexation, toxicity and enhanced pharmacokinetic performance [18,19].

Cyclodextrins offer several possibilities for constructing a LPV drug delivery system with enhanced bioavailability and biodistribution without the need of RTV. Apart from drug aqueous solubility and dissolution enhancement [20], CyDs have been reported to modulate CYPP450 metabolism and P-gp efflux transporters for enhanced drug bioavailability [21,22]. The need to achieve partial or complete inhibition of CYPP450 metabolism and P-gp efflux for enhanced drug permeability and pharmacokinetic performance has led to the incorporation of several CyD molecules into hybrid drug delivery systems [23,24]. However, in order to rationally design an efficient CyD based LPV delivery system, a detailed knowledge of its complex forming ability with different types of CyD molecules is required.

In this study, our aim was to study the LPV-CyD host guest molecular interaction and complex formation. An *in silico* method was developed and validated experimentally to screen different types of CyD in order to select the most optimal for LPV-CyD complex formation. The experimental validation involved the correlation of the rank order of experimentally derived association constants (phase solubility studies) of a model drug with different CyD molecules, with the rank order of the complexation energy derived from our *in silico* method. The hypothesis of our work is that the tedious and time/resource consuming traditional approach of selecting CyD by calculating association constants of a drug with several CyD molecules can be replaced with our *in silico* method. A derivative of (2-hydroxyl)propyl-gamma-cyclodextrin (HP-γ-CyD with a high degree of substitution - DS) was selected from the *in silico* study, synthesized and evaluated comparatively with parent γ-CyD and commercially available HP-γ-CyD. The complexes were prepared by supercritical fluid assisted spray drying (SASD) and co-solvent evaporation (CoEva) methods, and

afterwards, fully characterized to evaluate the influence of (2-hydroxyl)propyl derivatization/average degree of substitution (DS), formulation molar ratio and the method of preparation on LPV-CyD complex formation.

2. Materials and method

2.1. Materials

LPV (molecular weight (MW) 628.81 g/mol) was a kind gift from Mylan (India), while γ-CyD (MW~1297.12 g/mol) and HP-γ-CyD (CAVASOL®, DS = 4.6, MW~1540), were gifts from Wacker Chemie AG (Germany). HP17-γ-CyD (DS = 17.1, MW ~2324 g/mol) was synthesized in house. Ethanol (absolute 99.9% purity) was purchased from Carlo Erba Reagents. The water used in this study was purified with a Milli-Q water purification system (Water Max W1, Diwer Technologies)**.** Industrial carbon dioxide (purity ≥ 99.93%) was purchased from Air Liquide. All compounds were used as received.

2.2. *In silico* evaluation of LPV-CyD host guest molecular interaction

The initial molecular geometry of LPV was obtained from PubChem database (CID 92727) while those of the parent CyD molecules were retrieved from the Protein Data Bank (2ZYM, 3CGT and 2ZYK for α, β and γ-CyD respectively) and refined using the builder function on Molecular Operating Environment (MOE v2018.10, Chemical Computing Group, Canada). Since it is impractical to simulate all possible isomers or DS patterns of CyD derivatives, a set of 5 substitution models (*Supplementary information, SI, Table S1*) were simulated for each derivative using the most reactive secondary O2 and primary O6 sites of their glucopyranose unit [25,26]. The molecules were prepared by adding the hydrogen atoms at the right pH and their energies were minimized using the MMFF94 force field. In order to predict the most rational host-guest conformation of LPV-CyD (molar ratio 1:1), docking simulations were performed using a previously validated docking protocol. LPV and CyD were defined as the ligand and receptors respectively, with the flexibility of the ligand considered in opposition to the restriction of CyD flexibility which was defined as a rigid body. A maximum of 100 molecular ensembles of the ligand placed in the site with the Triangle Matcher method were saved and then ranked with the GBVI/WSA ΔG scoring function. The most energetically favourable binding poses were then chosen, the minimum energy mode of LPV, CyD and LPV-CyD complexes were computed and the complexation energy was calculated according to Eq. (1):

$$\Delta E = E_{complex} - \left(E_{host} + E_{guest}\right) \quad (1)$$

HP-γ-CyD Model B (*SI, Table S2*) with all the O2 and O6 positions of the parent γ-CyD glucopyranose units substituted (DS = 16) was selected as the CyD molecule with the highest potential for developing efficient LPV-CyD complexes and relabelled HP16-γ-CyD. Since this CyD derivative is not commercially available, and in order to facilitate further *in silico* (molecular dynamic, MD) comparison and experimental validation; a new Model CA (*SI, Table S1*) was simulated for the commercially available HP-γ-CyD (DS ≈ 4.6).

For the MD simulations, the most energetically favourable arrangements of γ-CyD, HP-γ-CyD, HP16-γ-CyD, and their complexes obtained from the docking studies were used. The structures were exported as PDB files and their topologies were generated using the Automated Topology Builder (ATB) and the Repository v.3.0 available on-line [27]. Energy minimizations and 100 ns MD simulations were performed with GROMACS 2016.03 program using the GROMOS-96 54a7 force field [28-31]. To eliminate interactions and promote periodic boundary conditions (PBC), the molecules of each system (*SI, Table S3*) was centered at the distance of 1.0 nm from the cubic box limit and solvated with water molecules using the SPC parameterization. Energy minimization using the steepest descent algorithm (<1000 steps) was performed to remove the unreasonable atomic contacts and stereo-chemical conflicts. Thereafter, two sequential NVT (1 ns at 300K) [32] and NPT (2 ps at 300K and 1 bar) simulations were carried out to stabilize the system [33]. These were followed by a 100 ns MD run with the default isothermal compressibility of water defined at 4.5×10^{-5} bar $^{-1}$. LINCS algorithm was used to achieve bond length constrain [34,35]. A cut-off of 1.0 nm was used to compute the short-range electrostatic and van der Waals interactions while the long-range electrostatic interactions were calculated using the Particle Mesh Ewald method [36,37]. The production runs were performed over 100 ns under the same conditions. The analysis was performed using modules available in GROMACS 2016.03 package. The dynamic behaviour and structure stability for all the systems were then interpreted.

2.3. Synthesis of HP-γ-CyD

The synthesis of the targeted HP-γ-CD was performed in three consecutive steps in order to minimize the formation of (oligo)propylene glycol and allow as much as possible, reaction of the secondary OH groups.

2.3.1. Step 1

Air-dry γ-CyD (0.015 mol, 19.5 g = 21.6 g air dry) was dissolved in NaOH (0.020 mol, 0.8 g) containing water (30 ml) and cooled below 5 °C using an ice-bath. When the temperature reached the value, (R/S)1,2-epoxypropane (0.100 mol, 5.8 g, 7 ml) was added under vigorous stirring. The ice-bath was changed to fresh ice which was allowed to melt (and not changed during the stirring period), and stirred for over the weekend. The reaction mixture turned to a suspension and after 90-120 min the suspension turned to a homogeneous solution. The reaction mixture was neutralized with strong cation exchanger (10 g, Amberlite® IR-120(H)) and stirred at room temperature (R.T) for 2 h, then filtered and washed with distilled water (4x20 ml). The light coloured solution was clarified with charcoal (2 g, 30 min/R.T.), filtration yielded a colourless clear solution, and the charcoal was washed with water (3x5 ml). The solution was freeze-dried and 24 g of white solid was obtained. To remove the (oligo)propylene glycols and tighten the substitution range, this crude product was dissolved in MeOH (24 ml) followed by the slow addition of acetone (240 ml) under sonication and using a spatula to get crystalline product. Filtration and washing with acetone afforded 21.0 g (89 %, based on DS = 4.6, determined by ^{1}H-NMR) after drying for the weekend at 70-80 °C in the presence of KOH. The dry sample can adsorb ≈5 % water at air after one day.

2.3.2. Step 2

The derivative (0.010 mol, 15.9 g) obtained in *Step 1* was dissolved in NaOH (0.020 mol, 0.8 g) containing water (20 ml) and cooled below 5 °C, then (R/S)1,2-epoxypropane (0.086 mol, 5.0 g, 6 ml) was added and the reaction was conducted as above. To the reaction mixture, saturated $NaHCO_3$ (8.4%, 35 ml) was added and then freeze dried. To remove the (oligo)propylene glycols, the obtained solid was treated with methyl-t-butyl ether (MTBE, 3x50 ml) and the dried solid gave a slightly yellow powder (25 g) which contained Na_2CO_3. This solid was boiled in MeOH (125 ml) for 30 min, then cooled, and the solids were removed by filtration and washed with MeOH (3x25 ml). Drying as above yielded very pale yellow powder (23.7 g). To remove residual bases the solid was dissolved in 100 ml MeOH and treated with strong cation ion exchanger (5 g, Amberlite® IR-120(H)) as above. Removal of MeOH from the filtration and ion exchanger washings (4x10 ml) afforded 20.4 g powder (103% calculated for DS = 12, but NMR analysis gave a DS of 11.4-12.4) with few O(2) substitution, (2-hydroxy)propoxy substituents, and (oligo)propylene glycol). The very pale yellow solid containing some MeOH was used without further drying or purification.

2.3.3. Step 3

HP γ-CyD derivative of *Step 2* (20.4 g) was dissolved distilled water (25 ml) containing NaOH (0.020 mol, 0.80 g) and cooled below 5 °C. Then, (R/S)1,2-epoxypropane (0.086 mol, 5.0 g, 6 ml) was added and the reaction was conducted as above. The (oligo)propylene glycol was removed and the product was neutralized as in *Step 2*. The obtained waxy solid (22.1 g) was dissolved in 40 ml water and dialyzed in a dialyzation cassette (MWCO 2k, Dial-A-Lyzer 2K G2 (ThermoFisher Scientific) in three portions against 550 ml water overnight. Freeze-drying of the dialyzed solution yielded an almost white, slightly hygroscopic powder (21.3 g, 90%, calculated DS = 17.1±1 by ^{1}H-NMR according to the Ph. Eur. 9 recommendation method), with minimal (oligo)propylene content (NMR). The product (HP17-γ-CyD) contains dominantly secondary O substituents as seen in the HSQC-DEPT spectrum (*SI. Figure S2*).

2.4. Preparation of solid binary products

The solid binary products of LPV with either of γ-CyD, HP-γ-CyD and HP17-γ-CyD (1.5 g each) were prepared in molar ratios of 1:1 and 1:2 using SASD and CoEva. Briefly, the accurately weighed quantities of LPV and CyD were dissolved in 50 % aq. EtOH and sonicated (Sonorex ultrasound bath; RK 100 H) for 20 min. The solutions were then filtered and pumped (2.5 ml/min; HPLC pump 305 Gilson) into a high-pressure static mixer saturator (3/16 model 37-03-075, Kenics Chemineer, 4.8 mm diameter, 191 mm length and 27 helical mixing elements) where a near equilibrium mixing occurred with supercritical carbon dioxide ($scCO_2$) (pumped at 25 ml/min; HPLC pump K-501, Knauer). The pressure (10.5 MPa) was measured by a Setra pressure transducer and the temperature of the mixture, at the static mixer (65 ^{o}C) was controlled by a Shinko FCS-13A temperature controller. The selection of operation parameters was based on previously optimized conditions in our laboratory [38]. The mixture was then atomized through a nozzle with an internal diameter of 150 μm into an aluminium precipitator operating at near-atmospheric conditions. At the same time, a flow of previously heated compressed air (T = 120 °C) entered the precipitator to evaporate the liquid solvent. The temperature measured at the exit of the precipitation chamber was around 70 °C. The formed particles were then separated from the Air-CO_2-solvent flow by the high efficiency Büchi cyclone. All powders were collected and stored in a screw capped glass

bottle prior to analysis. For the CoEva complexes, the filtered solutions were rotoevaporated and the recovered solids were dried overnight under vacuum.

2.5. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

AT-FTIR spectra were recorded on a PerkinElmer Spectrum Two Spectrometer (PerkinElmer, USA). Suitable amount of each powder was placed on the diamond plate surface, completely covering the surface of the prism. The spectrum was recorded (16 scans for each spectrum) from 400 to 4000 cm^{-1} with a resolution of 2 cm^{-1} and at room temperature.

2.6. X-ray powder diffraction (XRPD)

X-ray powder diffraction analysis was performed in a RIGAKU X-ray diffractometer (model Miniflex II) with automatic data acquisition (Peak search for Windows v. 6.0 Rigaku). Samples placed in a holder were analysed using CuK_α radiation (30 kV/15 mA) and the diffraction patterns were collected with a 2θ angle ranging between 5° and 55° and a scan rate of 0.02°/min.

2.7. ^{13}C Cross-polarization magic angle spinning CP/MAS nuclear magnetic resonance (NMR)

The solid-state ^{13}C CP/MAS NMR spectra were recorded on a Bruker NMR (Avance III 300 MHz WB) spectrometer using a 4 mm MAS multinuclear probe, operating at 75 MHz for ^{13}C. All spectra were recorded with an angular velocity of 5 kHz and 3500 accumulations. ^{13}C spectra were obtained with a TOSS (total suppression of spinning side bands) pulse sequence. The acquisition parameters were AQ (0.05 s); DW (10 us); DE (20 us), D1 (2 s); CP contact time (1200 us) and ^{1}H 90º pulse (4 us).

2.8. HPLC-MS/MS studies

The HPLC analyses of LPV were performed on a Waters Alliance 2695 (Waters®, Ireland) equipped with a quaternary pump, solvent degasser, auto sampler and column oven, coupled to a Photodiode Array Detector Waters 996 PDA (Waters®, Ireland). Chromatographic separation was achieved on a BDS-Hypersil 120 C18 (250 mm x 4.6 mm, 5 μm) column. The column temperature was set at 35 ^{o}C. An isocratic HPLC elution method consisting of 10% of eluent A (0.5% formic acid in Milli-Q water) and 90% eluent B (0.5% formic acid in

acetonitrile) was used. The flow rate was set at 0.3 ml/min and the injection volume was 10 µl. Tandem mass spectrometry (MS/MS) detection was performed on a Micromass® Quattro Micro triple quadrupole (Waters®, Ireland) using an electrospray ionization (ESI) source in positive mode (ESI+). Analytical conditions in the mass spectrometer were optimized in order to obtain the precursor and product ions of the compound.

2.8.1. Quantitative analysis of lopinavir

The analyses were performed in multiple reaction monitoring (MRM) mode in order to achieve a higher selectivity and sensitivity. The following transitions were used: MRM 1: 629.4>447.3 (quantification transition) and MRM 2: 629.4>183.2 (confirmation transition). Samples were diluted in MeOH before analysis (1:10 000, with a few exceptions, which were diluted 1:10 and 1:100). A calibration curve of LPV was prepared in MeOH, for each day of analysis, and ranged from 5 to 100 ng/mL. For data acquisition and processing, the software MassLynx® version 4.1 was used.

2.8.2. Drug content analysis

To determine the final LPV content in the complexes prepared by SASD and CoEva, accurately weighed amount of each powder was dispersed in 5 ml of 50% aq. EtOH and sonicated (Sonorex ultrasound bath; RK 100 H) for 25 min at 25 ^{o}C to allow for complete dissolution. The solutions were then filtered (0.2 µm) and assayed by HPLC-MS/MS The percentage of drug content was calculated as the ratio of assayed content to the initial LPV content in the formulation

2.8.3. LPV solubility measurements and drug release studies

LPV solubility in the presence of the three tested CyDs was determined by slight modification of a previously reported method [18]. Excess amount of pure LPV was added to 30% w/v phosphate buffer (pH 7.4) solutions of γ-CyD, HP-γ-CyD and HP17-γ-CyD. The dispersions were then kept in an orbital shaker incubator (IKA Shaker KS 4000 i control) at 37 ± 0.5 °C, 100 rpm, 24 hours. Final suspension was passed through a 0.2 µm syringe filter before evaluation by HPLC-MS/MS.

The *in vitro* LPV release from prepared complexes were determined by the dispersed amount method [39] in an orbital shaker incubator (IKA Shaker KS 4000 i control) maintained at 37

± 0.5 °C, 100 rpm, using a PBS pH 7.4 buffer as release medium (100µg LPV equivalent in 25 ml). At periodic intervals, samples were withdrawn from the release medium, syringe filtered and the amount of LPV released was assayed by HPLC-MS/MS.

3. Results and discussion

3.1. *In silico* studies and synthesis of HP-γ-CyD (high DS) derivative

In recent years, computational methodologies have been proposed as predictive tools for excipient selection in drug development [40,41]. Compared to the traditional experimental approach which is tedious, time and resource consuming; these tools can provide useful insights into the mechanistic relationships between active drug moieties and excipients at the molecular level. When using CyD for drug development, they can provide comprehensive atomic resolutions of the drug-CyD interactions and conformational properties, thereby facilitating a rational, deductive and knowledge based approach to drug development [42]. Herein, molecular docking was used to elucidate the LPV-CyD host-guest molecular interaction, and to select the CyD molecule with the best potential for developing a LPV-CyD drug delivery system. The molecular docking results and representative conformations (*SI, Table S2 and Figure S1*), suggest that LPV complex formation in molar ratio 1:1 is the most energetically favorable with a highly substituted HP-γ-CyD (DS= 16). Typically, the derivatization or addition of substituent groups to parent CyD enhances their intrinsic aqueous solubility and complex forming ability [43,44]. The number and type of substituent plays a fundamental role in altering the intrinsic physicochemical properties and functional behaviour of the resulting CyD derivatives [45,46]. While derivatization with large substituent groups can negatively impact complexation through self inclusion or occlusion of the CyD cavity, steric and electronic factors have also been reported to affect the complexation ability of CyD derivatives [18,19]. Our results (*SI, Table S2 and Figure S1*) suggest that this type of phenomenon, as most of the CyD derivative evaluated were thermodynamically unstable (positive complexation energy) compared to the parent β- and γ-CyD.

While molecular docking provides information on ligand-receptor interaction, its combination with molecular dynamics simulations allows us to understand the time-dependent/evolutionary nature of drug-CyD system equilibration and conformational stability in solution. Figure 3.1.2 shows root mean square displacement (RMSD), radius of gyrations

(Rg) and energy profiles of the LPV complexes of γ-CyD, HP-γ-CyD, HP16-γ-CyD, with respect to their initial docked structures. The energy profiles of the LPV-CyD complexes (Fig. 3.1.2a) suggest that HP16-γ-CyD is thermodynamically more stable compared to the commercially available HP-γ-CyD and γ-CyD (mean energy vales in *SI, Table S4).*

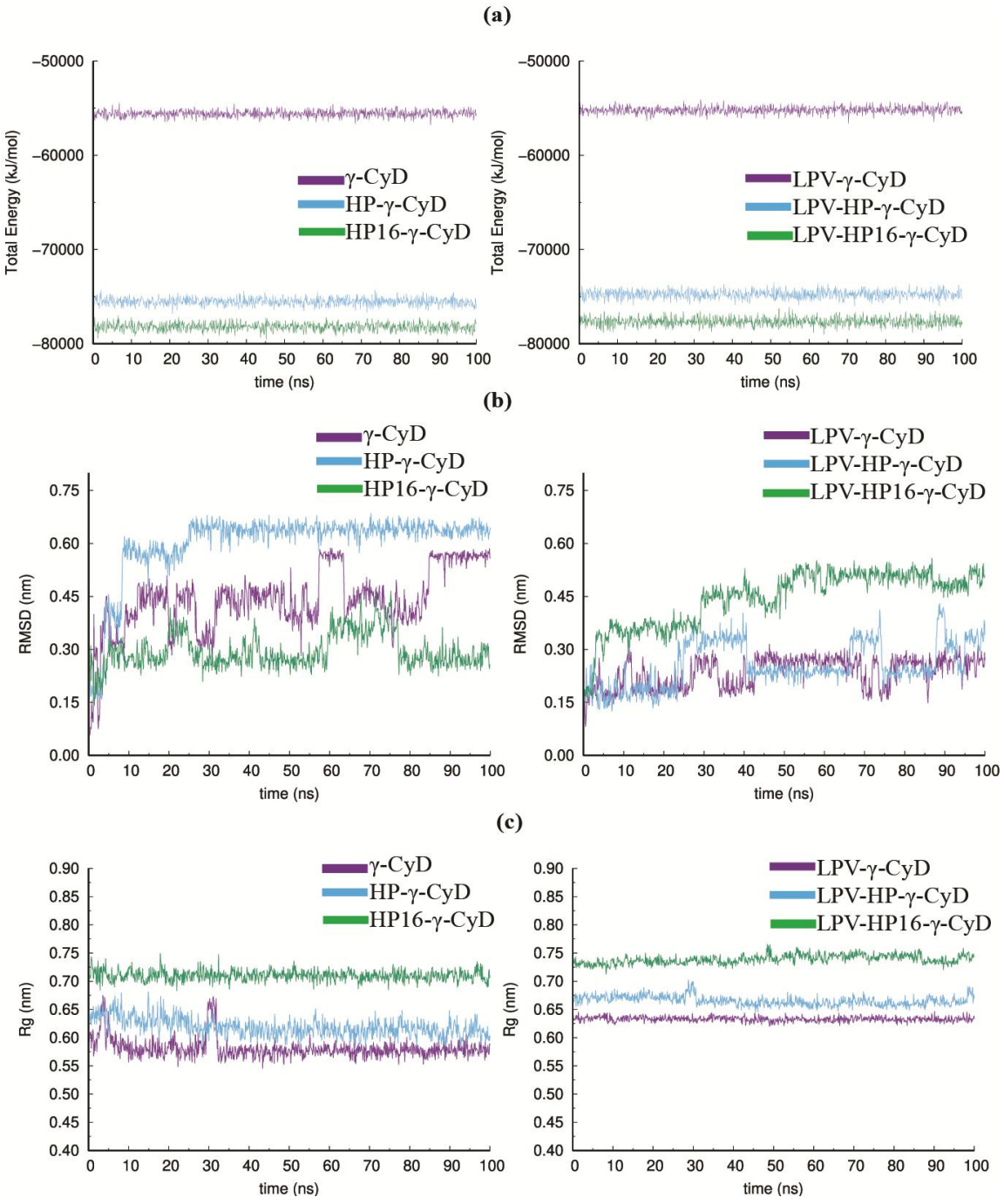


Figure 3.1.2. Plots of 100 ns Molecular Dynamics time-dependence/ evolution of (a) Total free energy (b) root mean square displacement (RMSD); and (c) radius of gyration (Rg).

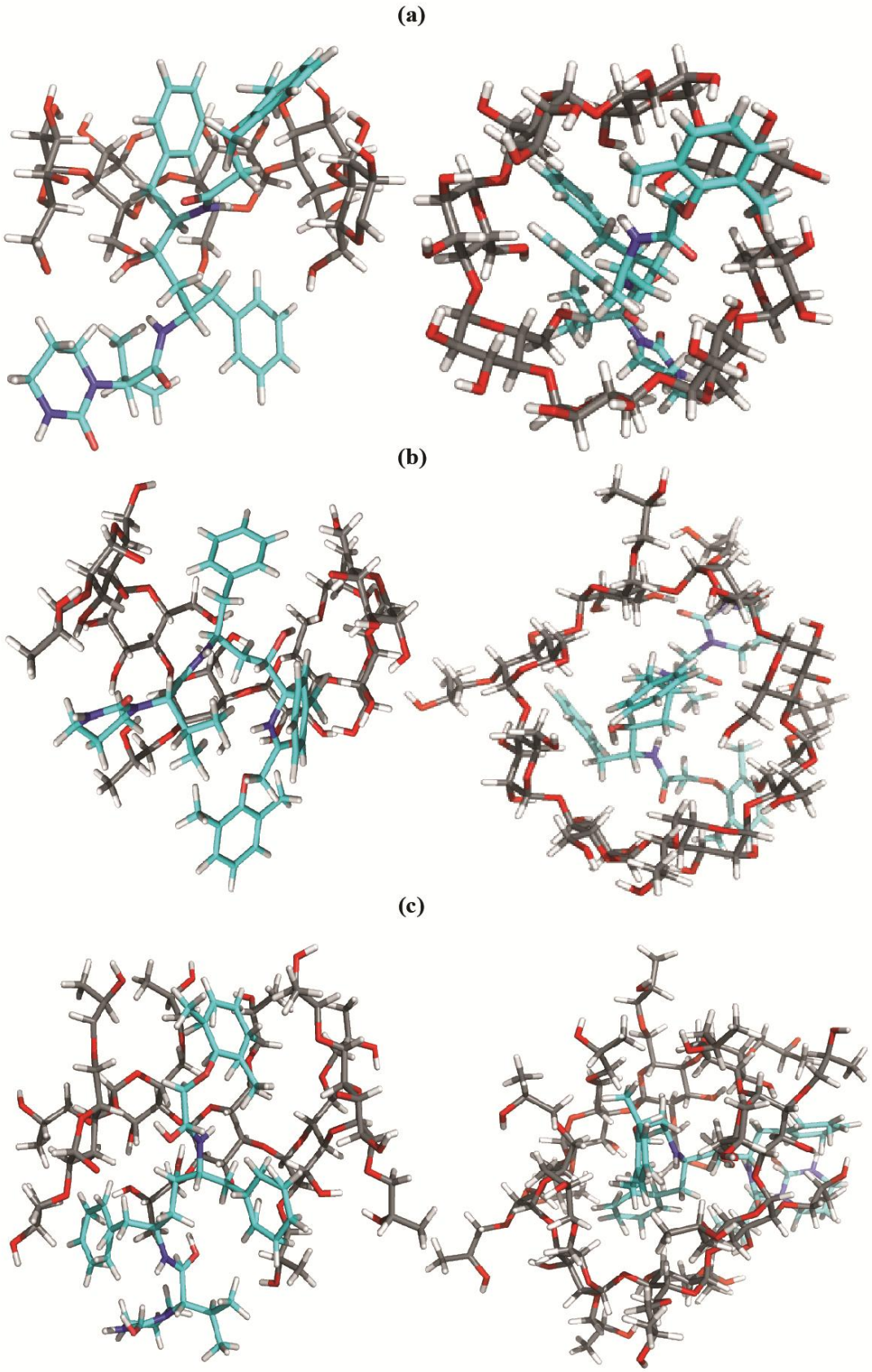


Figure 3.1.3 Front and top of LPV complexes snapshots at 100 ns (a) γ-CyD (b) HP-γ-CyD and (c) HP16-γ-CyD.

The system stability assessed by the RMSD (Fig 3.1.2b) was calculated along the 100 ns trajectories. Generally, the RMSD values after LPV-CyD complexes were lower than those of the initial structures indicating smaller structural variations in the LPV-CyD complex systems. The initial destabilized phase of the simulated systems (up to 40 ns) was followed by a more stable phase (> 40 ns) indicating the equilibrium was achieved. Also, complexes formed with HP16-γ-CyD were more stable than those of γ-CyD and HP-γ-CyD, suggesting that they are able to form stronger complexes with LPV. The radius of gyration of the LPV-CyD complexes were similar to those of initial structures suggesting that CyD molecules evaluated maintained their toroidal shapes even after complex system equilibrium was achieved. Figure 3.1.3 shows the 3D representations of the LPV-CyD complexes after 100 ns molecular dynamic trajectory. The orientation of LPV within the cavity CyDs suggests partial inclusion as some groups were outside the cavity. However, more groups were included the LPV-HP16-γ-CyD complex which has a larger cavity. Overall, both molecular docking and dynamics results suggest that HP-γ-CyD with a DS = 16 is the best CyD molecule to develop a CyD based LPV drug delivery system. Hence, this derivative was synthesized and confirmed by ^{1}H-NMR and HSQC-NMR analysis (*SI, Figure S2)*

3.2. Characterization of LPV-CyD complexes drug content

HPLC-MS analyses were performed to access the percentage of LPV present in prepared complexes (Figure 3.1.4). For all the prepared complexes, a minimum LPV content of 62.96 % was present.

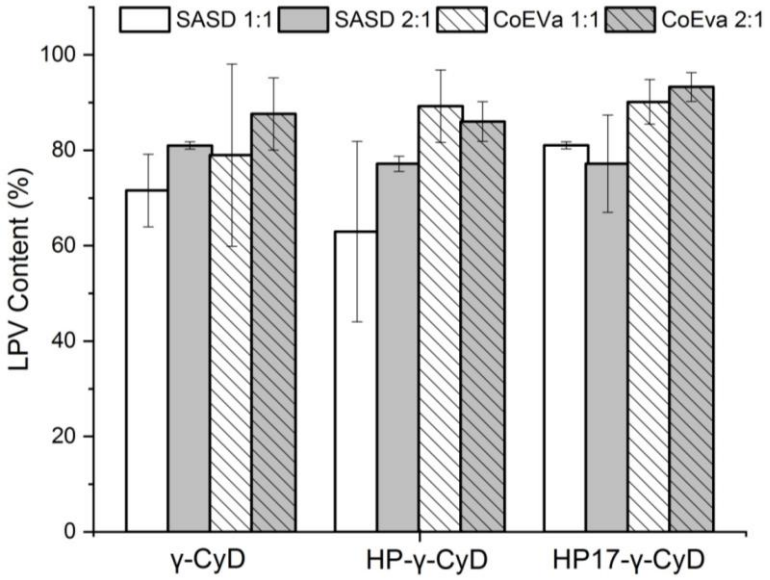


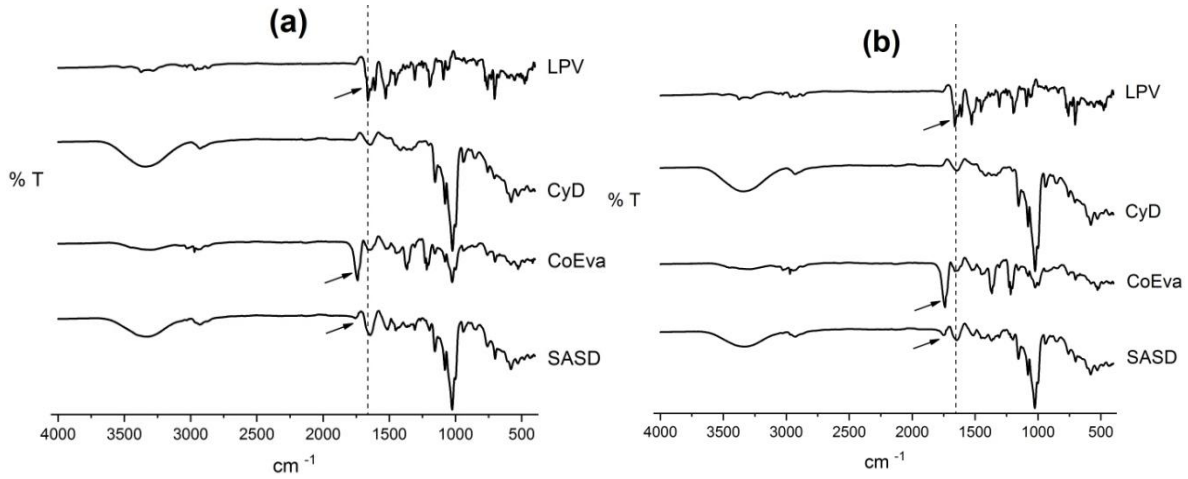
Figure 3.1.4. Percentage drug content in the LPV-CyD complexes.

The SASD prepared CyD complexes showed higher drug content (Figure 3.1.4) compared to those previously reported [38] indicating that SASD can be as efficient as co-evaporation in producing CyD complexes with high drug content.

3.3. Solid state characterization of SASD and CoEva complexes

3.3.1. ATR-FTIR

Host-guest interactions between LPV and each of γ-CyD, HP-γ-CyD and HP17-γ-CyD were studied using ATR-FTIR. Typically, as the guest molecule attempts to fit itself into CyD cavity, the restriction of its stretching vibrations and/or weakening of its interatomic bonds changes the position, shape and intensity of its absorption bands. These changes provide insight into the host-guest interaction at the molecular level, the formation of complexes and their nature [38,47,48]. Figure 3.1.5(a-f) shows the ATR-FTIR spectra of uncomplexed LPV, pure CyDs and the prepared complexes. The spectrum of LPV showed characteristics peaks at 3371 cm^{-1}, 3277 cm^{-1} and 1661 cm^{-1} corresponding to the stretching vibrations of OH, NH and CO (amide groups) respectively. The carbonyl groups of LPV are excellent candidates to show distinct variations attributable to complexation with CyD since its OH and NH absorption bands are easily masked by the broad prominent stretching vibrations of CyD's primary and secondary OH groups between 3000 and 3600 cm^{-1}. Figure 3.1.5 shows that LPV's carbonyl bands shifted to higher frequencies in all the prepared complexes, suggesting the dissociation of intermolecular hydrogen bonds associated with crystalline drug molecules due to molecular interaction and complex formation with CyD [49]. This shift to higher frequencies was not observed in physical mixtures of LPV and each of the tested CyD (*SI, Figure S3*).



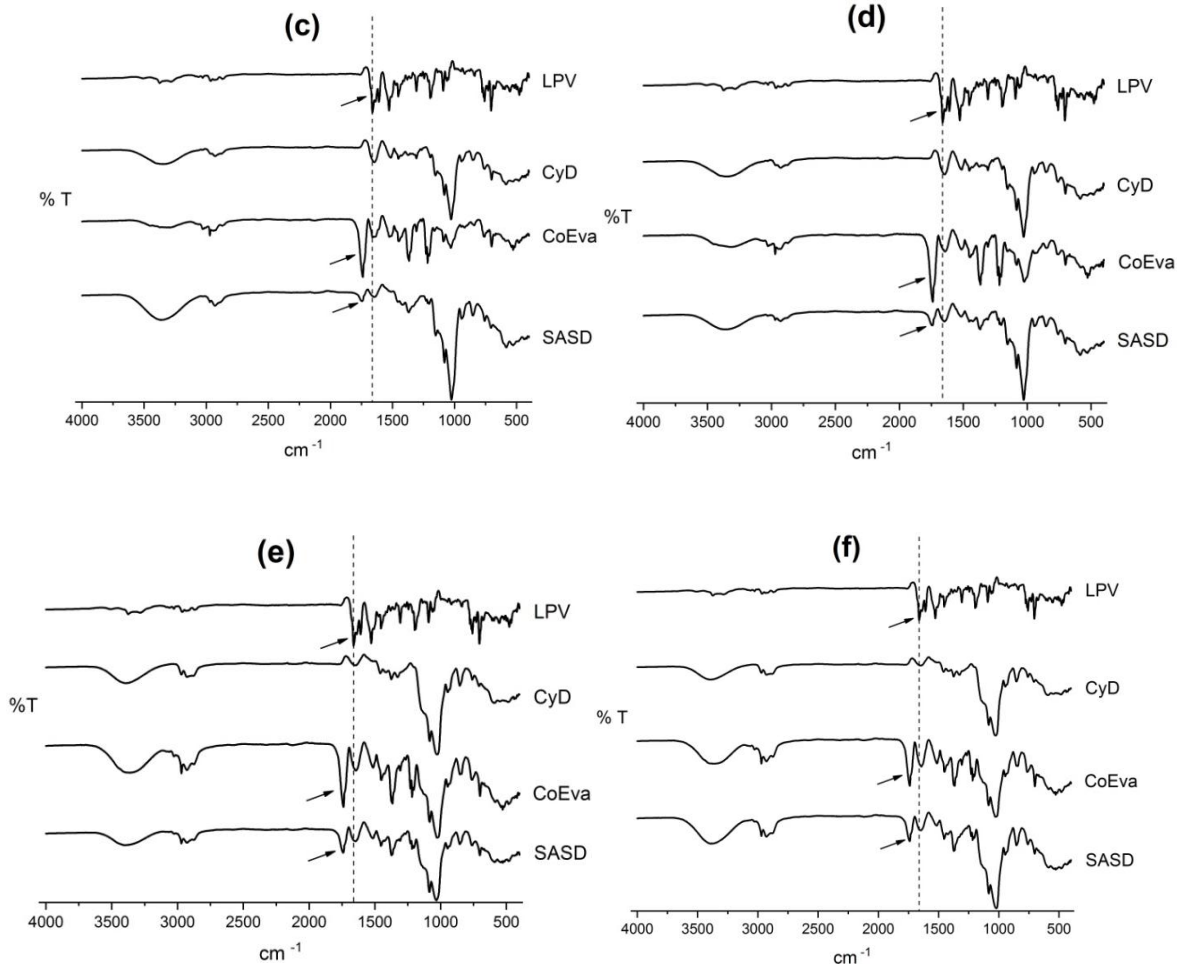


Figure 3.1.5. ATR-FTIR spectra of (a) γ-CyD 1:1 (b) γ-CyD 2:1 (c) HP-γ-CyD 1:1 (d) HP-γ-CyD 2:1; (e) HP17-γ-CyD 1:1; and (f) HP17-γ-CyD 2:1

Also, complexes prepared by SASD showed a higher reduction in the carbonyl band intensities compared to those prepared by CoEva. This reduction has been suggested to be due to the close fitting of the carbonyl groups within CyD cavity consequently hindering their bending and reducing the intensities of their vibration modes [50].

3.3.2. XRD

The XRD patterns of LPV, γ-CyD, HP-γ-CyD, HP17-γ-CyD and their solid complexes are shown in Figure 3.1.6(a-f). The diffraction analysis of LPV shows sharp and intense peaks indicating a crystalline nature of the drug [14], while those for γ-CyD, HP-γ-CyD and HP17-γ-CyD revealed a diffused-halo pattern indicative of their amorphous nature. Typically, CyD induced changes in drug crystallinity occurs as a consequence of solid state interaction with

the drug. While complete amorphization has been suggested as evidence on complete inclusion complex formation [51], this can only be used as complementary evidence in addition to other analytical methods because of the independent effect of methods of CyD complex preparation on crystalline drug amorphization [48].

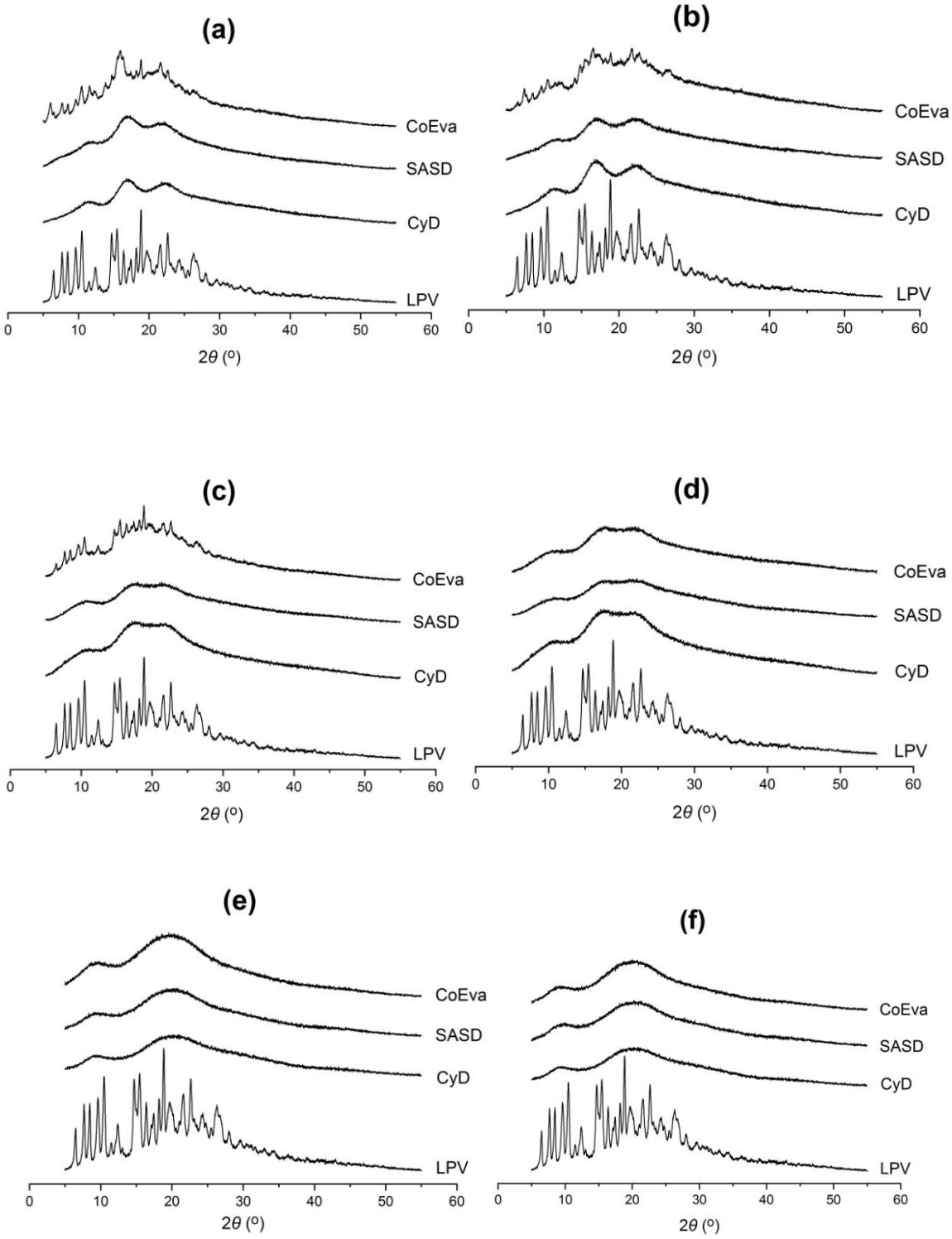


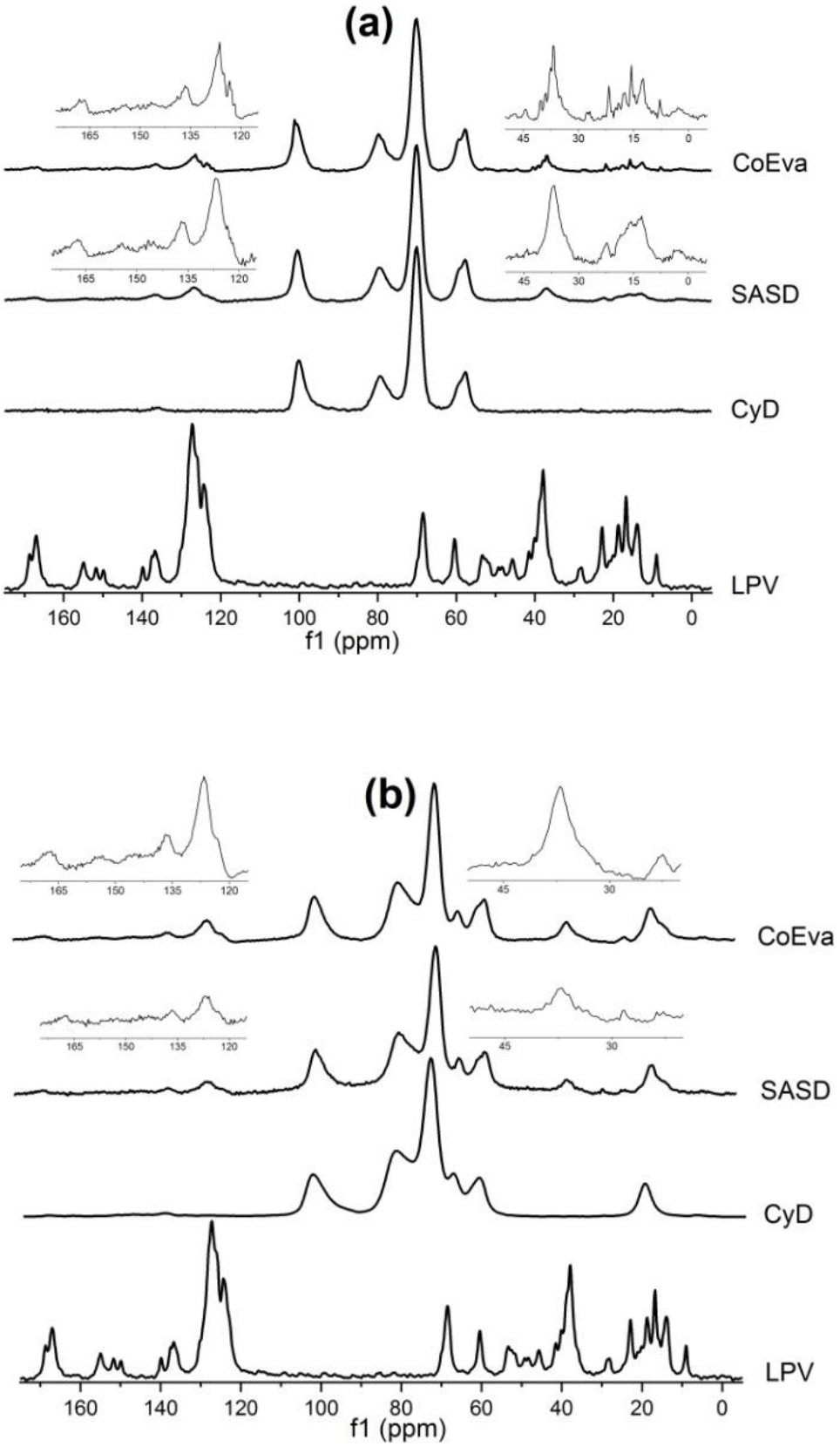
Figure 3.1.6. X-ray diffractograms of (a) γ-CyD 1:1 (b) γ-CyD 2:1 (c) HP-γ-CyD 1:1 (d) HP-γ-CyD 2:1 (e) HP17-γ-CyD 1:1; and (f) HP17-γ-CyD 2:1

For instance, the method chosen for the preparation of CyD complexes can affect the extent of inclusion complex formation and/or amorphization [52]. For all the complexes prepared by SASD, a complete amorphization of LPV was observed. However, for complexes prepared by CoEva, the degree of LPV crystallinity or amorphization was affected by the type of CyD used and molar ratio of such mixtures. The intensity of LPV's crystalline peaks was reduced in γ-CyD complexes suggesting incomplete amorphization, while for HP-γ-CyD, complete amorphization was dependent on the CyD-LPV molar ratio (found only CyD-LPV with molar ratio of 2:1). For co-evaporated complexes of HP17-γ-CyD, complete amorphization was found at both molar ratios evaluated. Since higher degrees of crystalline drug amorphization has been correlated with increase in aqueous solubility/dissolution [53-55] this results suggests that newly synthesized HP17-γ-CyD would facilitate a higher degree of LPV amorphization as compared to γ-CyD and HP-γ-CyD. The complete amorphization observed in all SASD complexes also suggest that it is a more effective method (compared to the CoEva method) for LPV-CyD complex formation.

3.3.3. ^{13}C CP/MAS NMR

The solid state ^{13}C CP/MAS NMR spectra of LPV, γ-CyD, HP-γ-CyD and HP17-γ-CyD and their complexes are presented in Figure 3.1.7(a-c). The well-resolved and sharp ^{13}C resonances of LPV signifying a crystalline system are similar to those previously reported in literature [55]. For the CyD molecules, the ^{13}C signals were also similar to those previously reported in literature [38,56,57] with newly synthesized HP17-γ-CyD showing more intense (2-hydroxy)propyl ^{13}C signals as compared to HP-γ-CyD. Following complex formation, most of the crystalline ^{13}C resonance signals of LPV disappeared while those that remained showed a preparation method dependent signal intensity reduction and broadening. As shown in Figure 3.1.7, the SASD prepared complexes showed more broadened and less intense signals as compared to CoEva prepared samples. This is indicative of new interactions between LPV and the evaluated CyDs and also consistent with the XRD results which suggested that SASD is more effective than CoEva in the amorphization of LPV. Both γ-CyD and HP17-γ-CyD showed no significant shifts in ^{13}C signals after LPV complexation and are thus in agreement with the MD results (Figure 3.1.2) about the retention of local conformation after complexation. Conversely, HP-γ-CyD showed approximately 3 ppm upfield shift in its ^{13}C signals suggesting a more pronounced influence of LPV on the

dihedral angles of its glycosidic α(1→4) bonds and the torsion angles which describes the orientation of its hydroxyl or



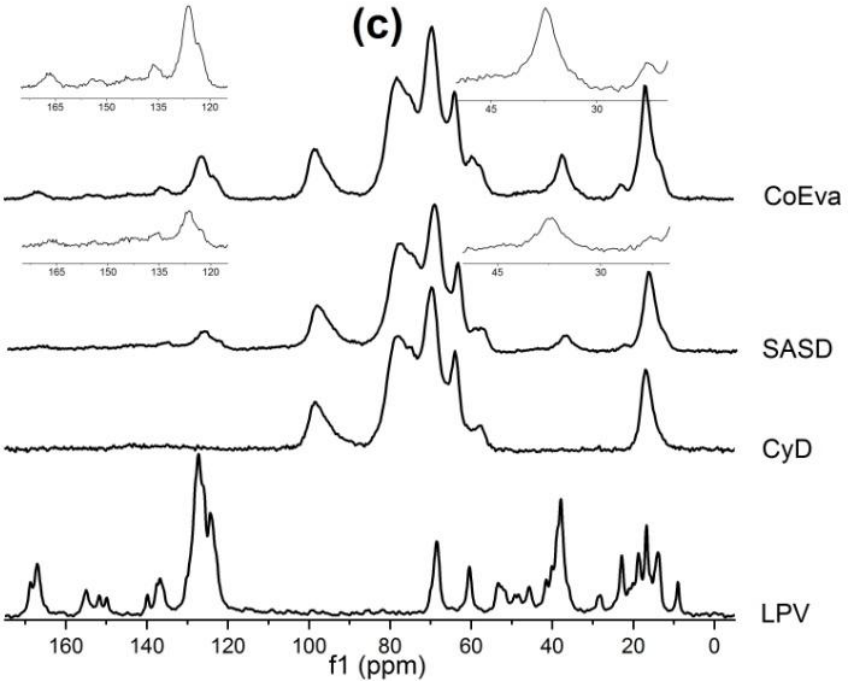


Figure 3.1.7. ^{13}C CP/MAS NMR spectra for complexes with molar ratio (2:1) (a) γ-CyD (b) HP-γ-CyD (c) HP17-γ-CyD (inserts show the magnified region of the NMR spectra)

substituent groups [58,59]. It is important to note that; unlike the solid state ^{13}C CP/MAS NMR analysis, the MD simulations occurs in a solvated system (*SI, Table S3*) where LPV and CyD are in equilibrium and slight changes in CyD's ^{13}C signals local conformation can be averaged over the 100 ns trajectory. The conformation of LPV in the CyD cavities (Figure 3.1.3 and *SI, Figure S5*) also suggests a more constrained LPV orientation within the HP-γ-CyD structure. It is possible that a higher electronic density exerted LPV's attempt to fit its cyclic urea moiety, secondary amide groups and one of its benzene rings into HP-γ-CyD cavity may have played a role in the observed differences ^{13}C signals shifts. Due to differences in cavity dimensions, these functional groups were outside the cavity of γ-CyD while the larger cavity size of HP17-γ-CyD allowed for a less constrained LPV conformation (*SI, Figure S5 and Table S7).*

3.4. Lopinavir solubility measurements and *in vitro* drug release

HPLC-MS/MS analyses were performed in order to quantify LPV solubility and *in vitro* drug release. As shown in Figure 3.1.8a, CyD derivatization generally increased LPV solubilization with γ-CyD, HP-γ-CyD and HP17-γ-CyD resulting in a 87, 114 and 129 fold

increase, respectively. As predicted by *in silico* studies, the newly synthesized HP17-γ-CyD solubilized more LPV than parent γ-CyD and HP-γ-CyD. This suggests that the cavity size increase observed in HP17-γ-CyD (SI, Table S7) occurred in a way that is complimentary for the formation of LPV complexes i.e. without steric hindrances. This suggests that the CyD cavity size increased without steric hindrances in a way that is complimentary for the formation of complexes with LPV [60]. This conclusion is also confirmed by the drug release profile (Figure 3.18(b-c)) compared on the basis of Q_{60} (i.e. percentage of drug dissolved in 60 min) which showed higher values for HP17-γ-CyD relative to HP-γ-CyD and γ-CyD. However, the burst effect typically observed for CyD complexes was not observed even though HP17-γ-CyD showed higher amount of drug dissolved in the first 15 minutes. Also, the SASD processing technology contributed to the higher solubility relative to CoEva.

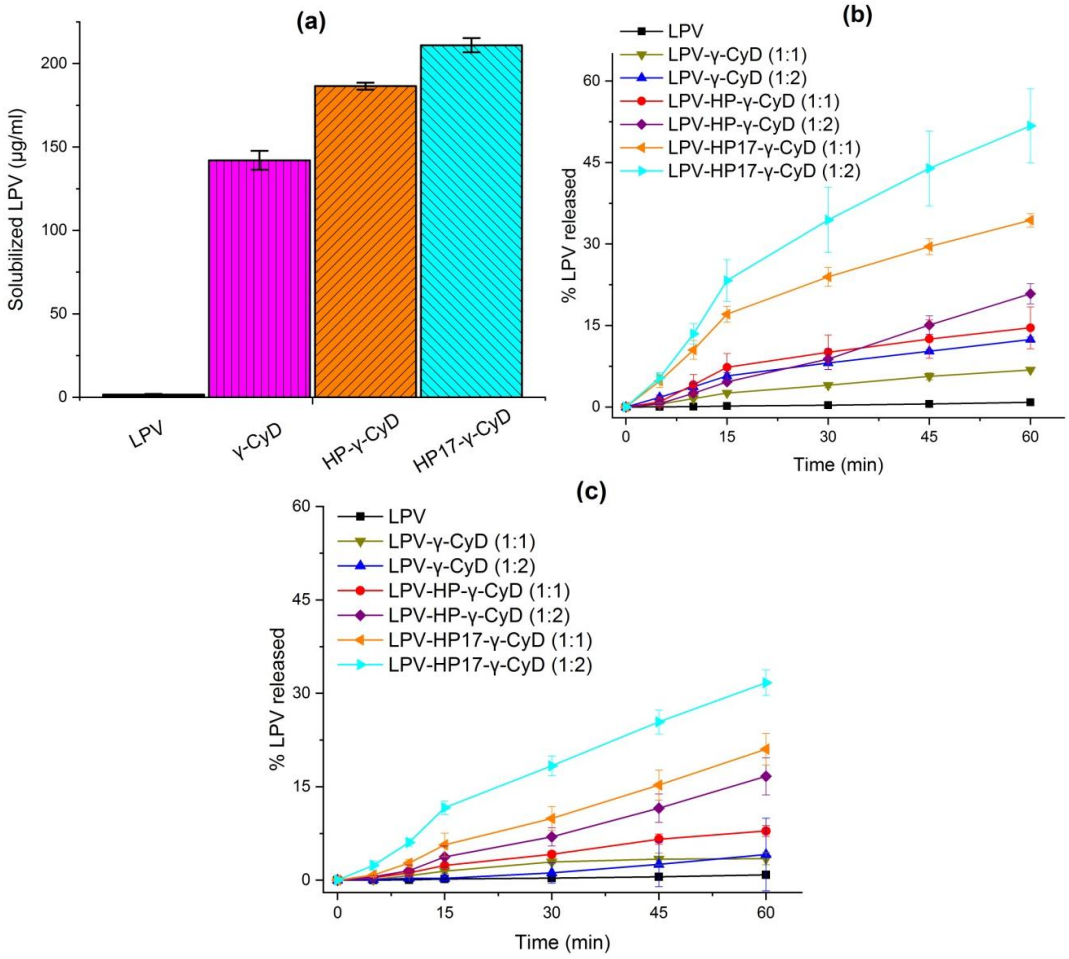


Figure 3.1.8. (a) Effects of (2-hydroxyl)propyl derivatization and average degree of substitution on the solubility of LPV (b) drug release from SASD prepared complexes; and (c) drug release from CoEva prepared complexes

It is important to note that LPV release from SASD prepared HP17-γ-CyD complexes after 15 min (51.7%) is significantly higher than the values reported by Goyal and Vavia who studied LPV release from γ-CyD complexes under sink conditions (≈ 12 %) [20]. This highlights the utility of incorporating computational methodologies as predictive tools for CyD selection for the preparation of CyD based drug delivery systems.

4. Conclusion

This study demonstrated the utility of computational methodologies as a predictive tool for CyD selection in the development of CyD based drug delivery systems. Using molecular docking and dynamics, it was possible to evaluate several factors implicated in CyD host guest molecular interaction with LPV and to select the best CyD for preparing LPV complexes. It was also possible to completely avoid the tedious and time/resource consuming traditional approach of selecting CyD by calculating association constants from phase solubility studies or isothermal titration calorimetry. The *in silico* method allowed us to focus on pre-identified and less tedious endpoint physicochemical properties such as drug solubilization. The result obtained from ^{1}H-NMR and HSQC-NMR analysis confirms the successful synthesis of the predicted (2-hydroxyl)propyl derivative (HP17-γ-CyD) while use of solid state analytical techniques such as AT-FTIR, XRD, ^{13}C CP/MAS NMR and the combined evaluation of their results revealed a higher LPV amorphization ability of HP17-γ-CyD relative to the parent γ-CyD and the commercially available HP-γ-CyD. Solubility measurements and drug release studies also revealed the higher ability of HP17-γ-CyD to facilitate LPV solubilization. Also, SASD processing technique enhanced LPV complexation and solubilization. Apart from the traditional pharmaceutical application of CyD in enhancing drug aqueous solubility, physicochemical and physiological stability, and the *in-vivo* deliverability; CyDs are increasingly being used in the development of complex hybrid drug delivery systems. Within this context, the application of *in silico* methodologies is a feasible approach for the rational and/or deductive development of CyD drug delivery systems.

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6. Supporting Information

Table S1: Model Summary for *in silico* CyD derivatives.

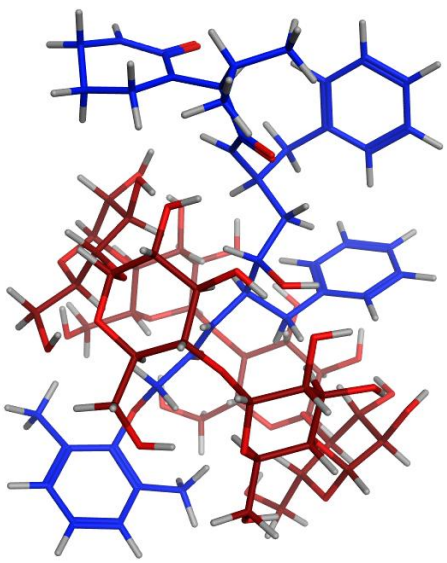
CyD Derivative	Substitution	Models	Substitution position	
			O2	O6
(2-hydroxy)propyl-beta-CyD (HP-β-CyD)	$CH_2CHOHCH_3$	A	All	None
		B	All	All
		C	All	1,3,5,7
		D	1,3,5,7	1,3,5,7
		E	None	All
Sulfobutyl Ether Beta CyD (SBE-β-CyD)	$(CH_2)_4SO_2$	A	All	None
		B	1,3,5	2,4,6
		C	2,4,6	1,3,5
		D	1,3,5,7	2,4
		E	None	All
(2-hydroxy)propyl-gamma-CyD (HP-γ-CyD)	$CH_2CHOHCH_3$	A	All	None
		B	All	All
		C	All	1,3,5,7
		D	1,3,5,7	1,3,5,7
		E	None	All
(2-hydroxy)propyl-gamma-CyD (HP-γ-CyD) commercially available	$CH_2CHOHCH_3$	CA	1,3,5	2,6

Table S2: LPV-CyD complexation energy.

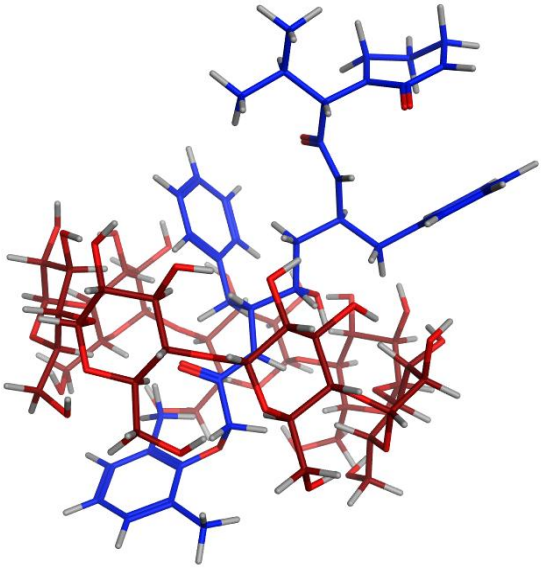
CyD	Docking Score (S)	ΔE
α-CyD	-4.982	26.99
β-CyD	-5.52	-2.02
γ -CyD	-6.09	-3.76
HP-β-CyD Model A	-6.805	12.83
HP-β-CyD Model B	-5.054	1.31
HP-β-CyD Model C	-6.699	14.23
HP-β-CyD Model D	-6.647	1.82
HP-β-CyD Model E	-6.851	16.84
SBE-β-CyD Model A	-6.691	14.41
SBE-β-CyD Model B	-7.127	4.69
SBE-β-CyD Model C	-6.689	8.39
SBE-β-CyD Model D	-7.265	14.83
SBE-β-CyD Model E	-6.282	14.05
HP-γ-CyD Model A	-7.390	6.00
HP-γ-CyD Model B	-6.889	-10.09
HP-γ-CyD Model C	-7.076	6.87
HP-γ-CyD Model D	-6. 326	7.23
HP-γ-CyD Model E	-6.485	-0.47

Table S3: Cell compositions and atom number for the complexes and the number of water molecules used for the molecular dynamics simulations.

	γ-CyD	LPV-γ-CyD	HP-γ-CyD	LPV- HP-γ-CyD	HP*16*-γ-CyD	LPV-HP*16*-γ-CyD
Water shell volume (Å)	51.46 $x10^3$	56.94 $x10^3$	70.30 $x10^3$	73.96 $x10^3$	73.55 $x10^3$	81.04$x10^3$
Atom number of LPV	0	67	0	67	0	67
Atom number of CD	151	152	177	177	232	232
Molecule number of waters	1649	1637	2237	2212	2325	2307



(a)



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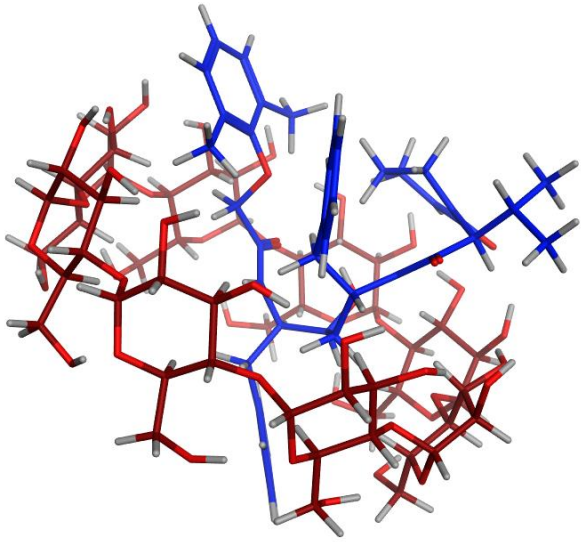

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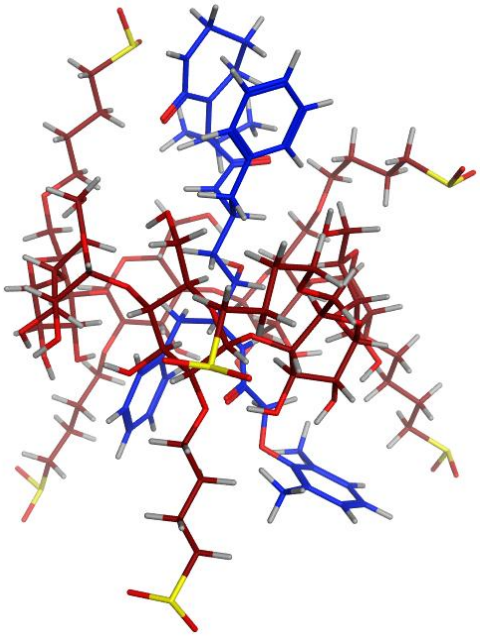

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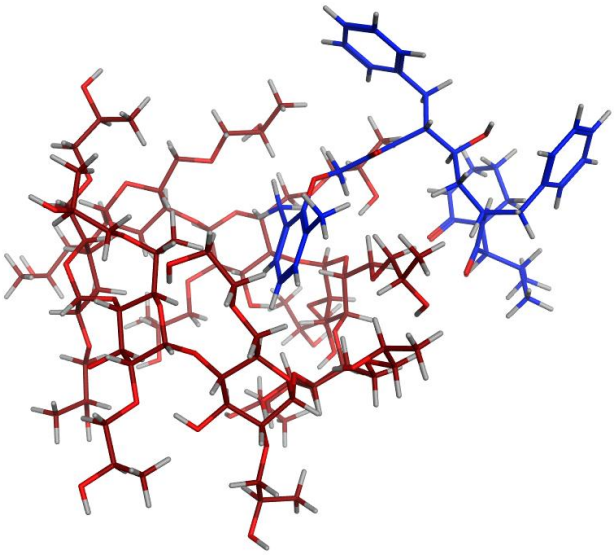
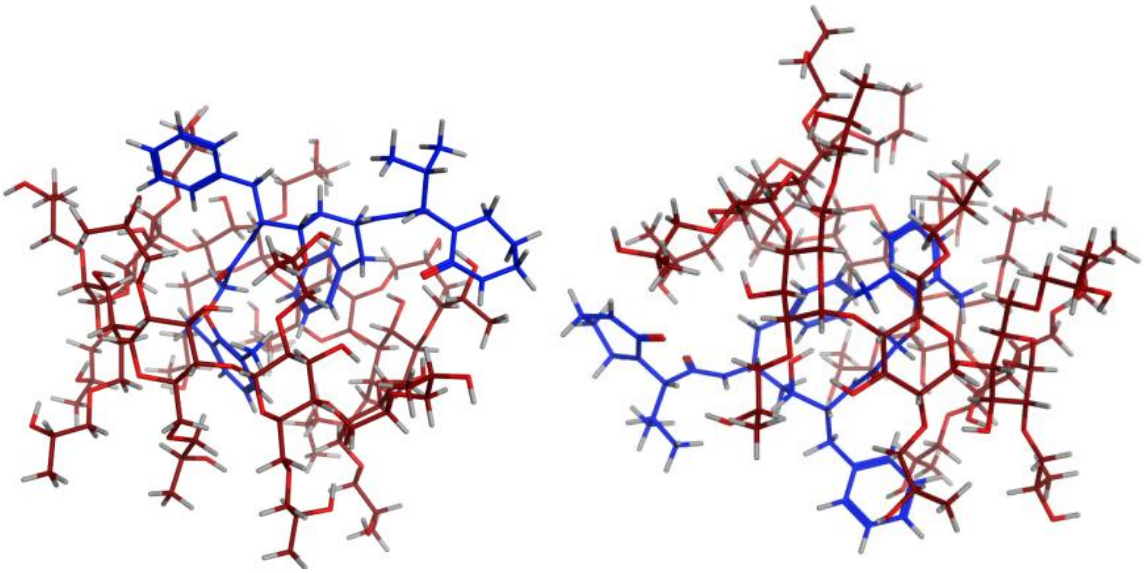


(e)



(f)

Figure. S1 Representative docking conformations of: (a) LPV-α-CyD (b) LPV-β-CyD (c) LPV-γ-CyD (d) LPV-SBE-β-CyD (e) LPV-HP-β-CyD (f) LPV-HP-γ-CyD (two views)

Table S4: Mean energy values of the molecular dynamics simulations over 100 ns.

CyD and LPV complexes	Mean energy values	Change in Mean energy after complexation (ΔE)
LPV	-57165.0 ± 2.6	-
γ-CyD	-55581.5 ± 1.6	372.3
LPV-γ-CyD	-55209.2 ± 1.8	
HP- γ-CyD	-75557.6 ± 2.1	767.5
LPV-HP-γ-CyD	-74790.1 ± 5.8	
HP16- γ-CyD	-78219.1 ± 0.91	555.6
LPV-HP16- γ-CyD	-77663.5 ± 4.2	

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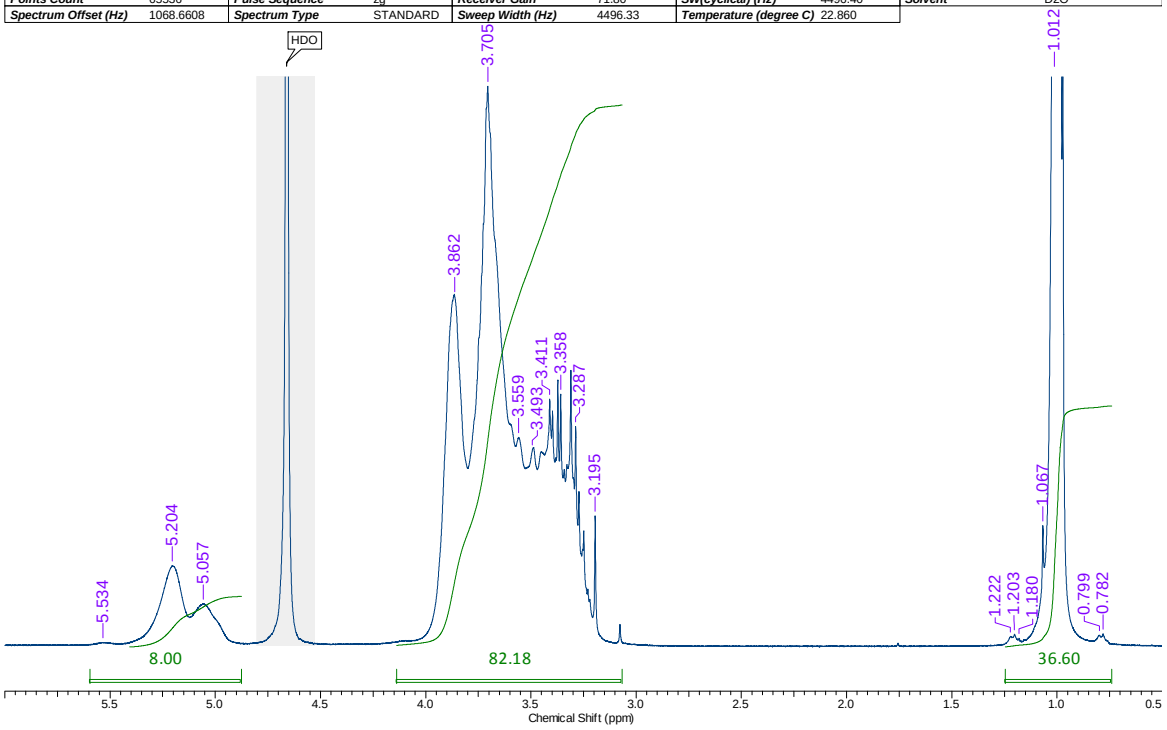

(a)

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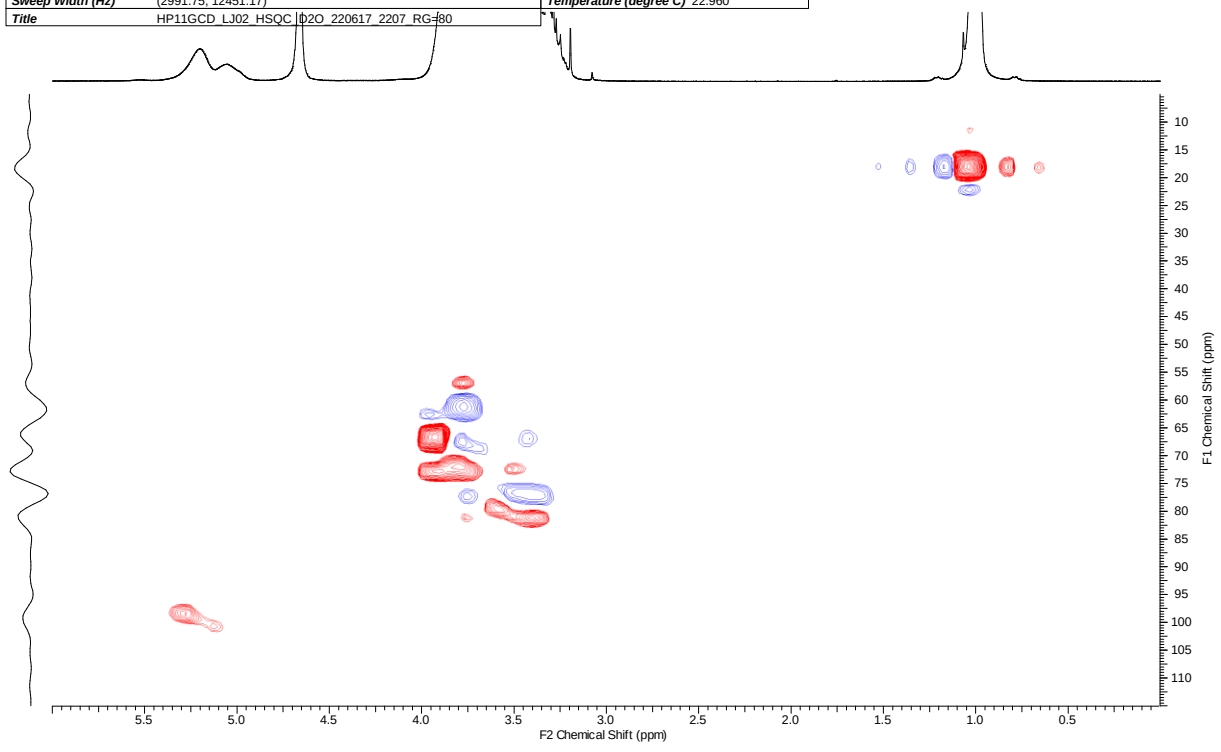

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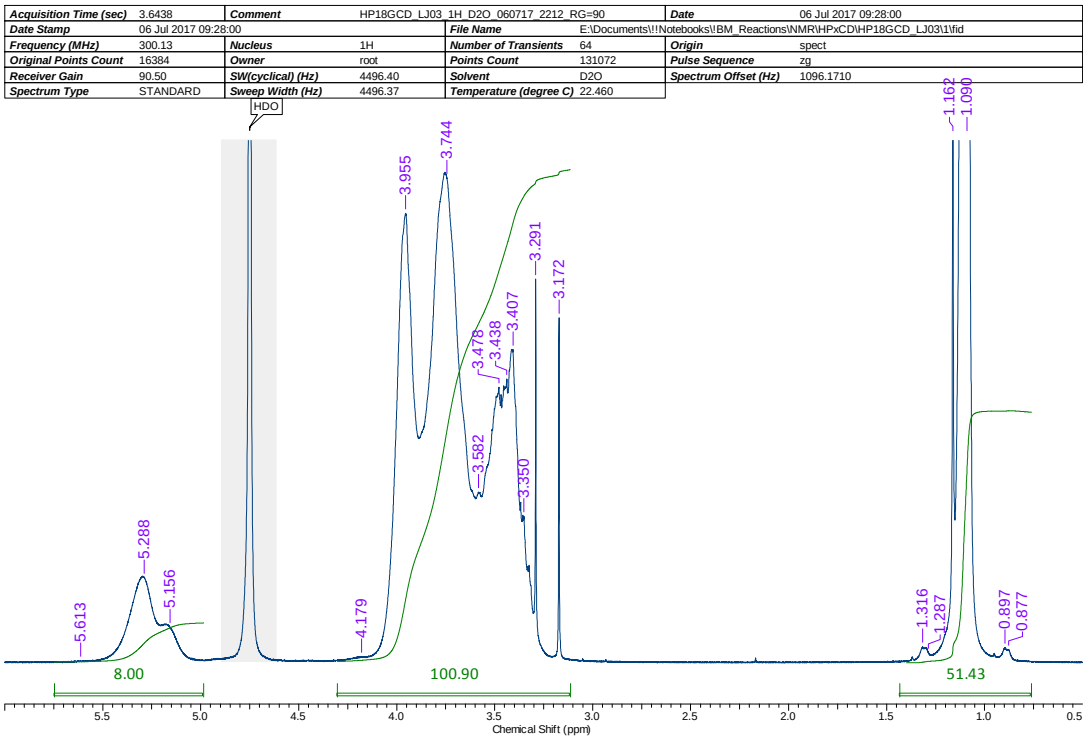

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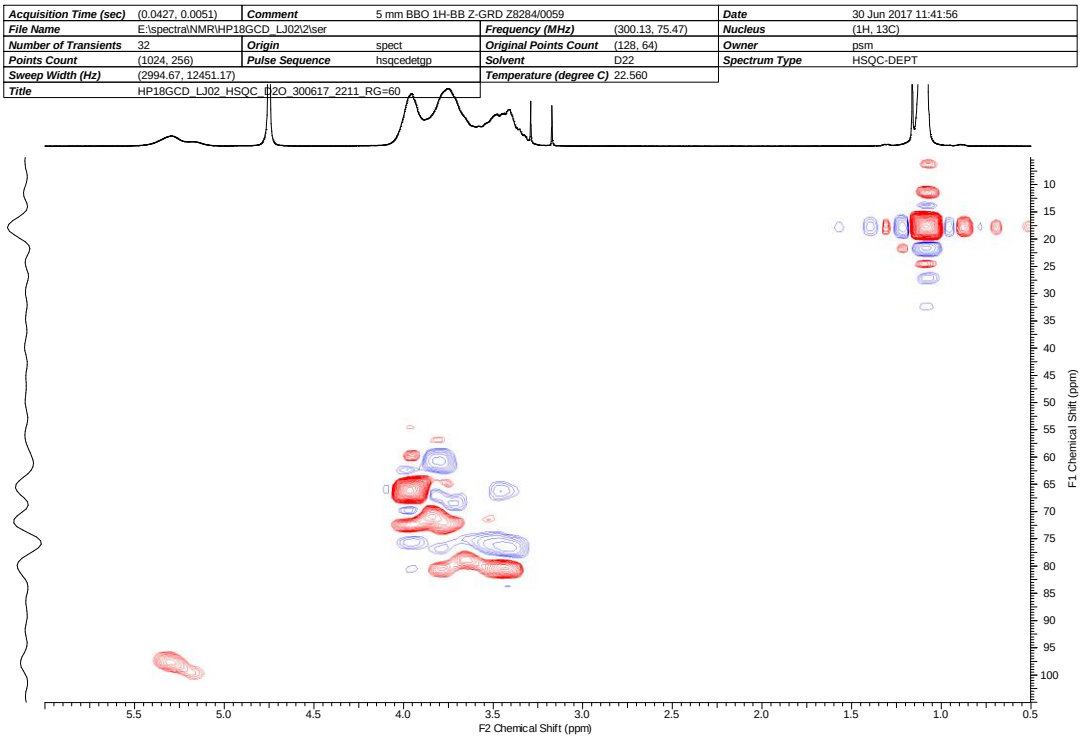


(d)

Figure S2. (a, b) ^{1}H-NMR spectrum for intermediate HP12-γ-CyD and HP17-γ-CyD respectively; and (c, d) HSQC-NMR spectrum for intermediate HP12-γ-CyD, and HP17-γ-CyD, respectively

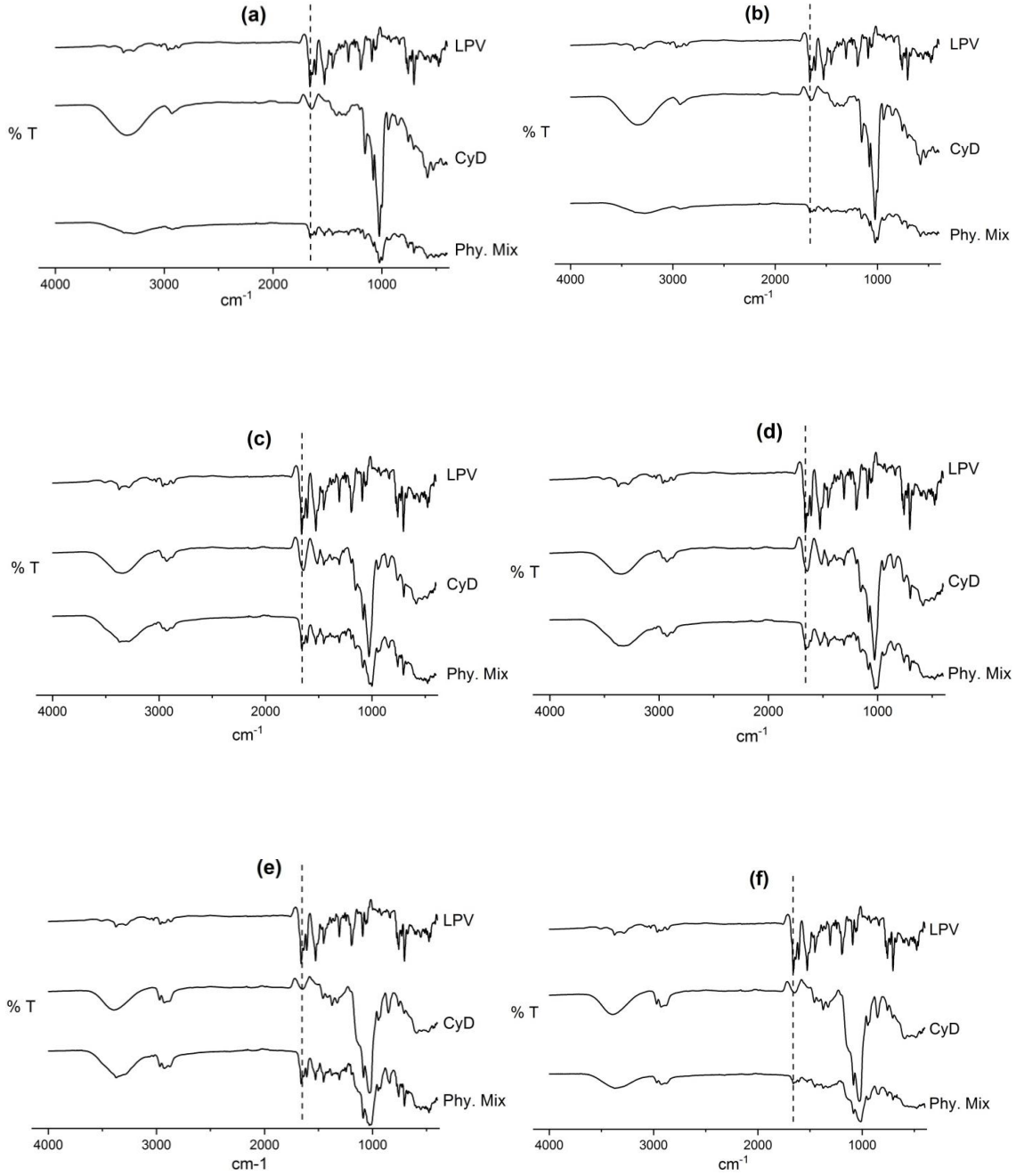
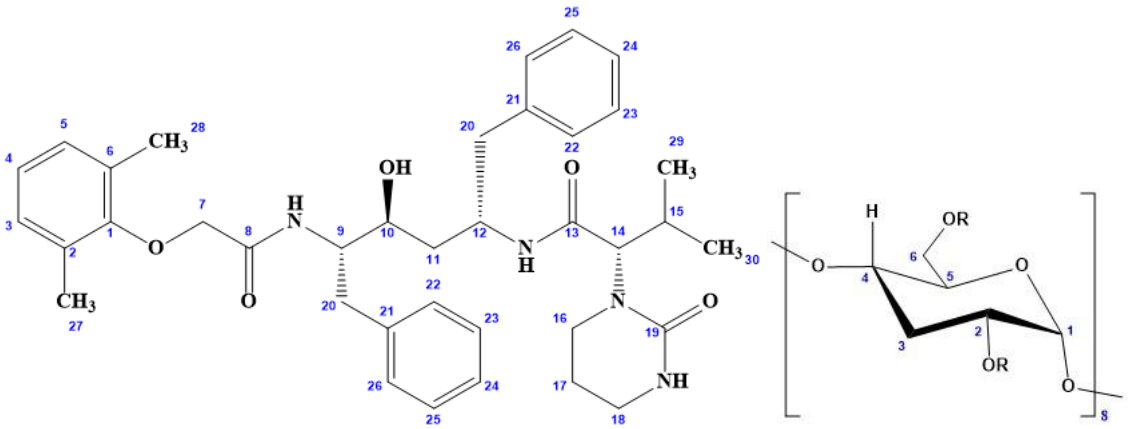


Figure S3. AT-FTIR spectra of physical mixtures for (a) γ-CyD 1:1 (b) γ-CyD 2:1 (c) HP-γ-CyD 1:1 (d) HP-γ-CyD 2:1 (e) HP17-γ-CyD 1:1 (f) HP17-γ-CyD 2:1



(a) **(b)**

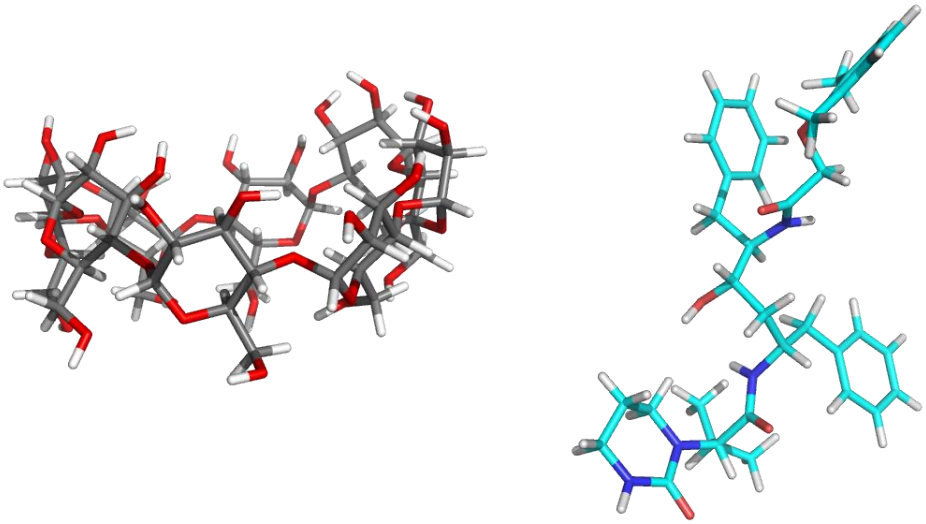
Figure S4. Carbon number assignments for (a) Lopinavir and (b) Cyclodextrin

Table S5: The ^{13}C CP/MAS NMR chemical shifts (δ, ppm) obtained from CyD and their LPV complexes.

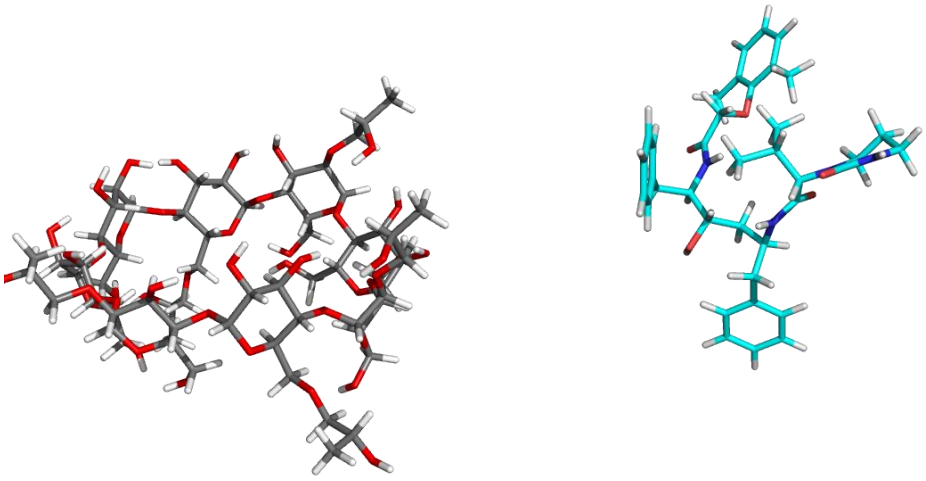
CyD and LPV-CyD complexes	Carbon number				Carbon number for group substituents	
	1	2,3,5	4	6	R1	R2
γ-CyD	100.15	70.09	79.37	57.61		
LPV- γ-CyD (SASD)	100.14	70.05	79.20	57.75		
LPV- γ-CyD (CoEva)	101.02	70.13	79.70	57.67		
HP-γ-CyD	103.07	73.32	82.14	60.99	67.63	19.63
LPV-HP- γ-CyD (SASD)	99.85	69.93	79.13	57.69	64.12	16.02
LPV-HP- γ-CyD (CoEva)	99.80	69.94	79.09	57.49	64.14	16.04
HP17-γ-CyD	98.34	69.63	78.03	57.63	63.89	16.83
LPV-HP17-γ-CyD (SASD)	98.62	69.65	78.61	59.84, 58.27	63.92	16.83
LPV-HP17-γ-CyD (CoEva)	97.97	69.46	78.08	59.63	63.82	16.80

Table S6: The ^{13}C CP/MAS NMR positions (δ, ppm) for crystalline Lopinavir.

Carbon number	(δ, ppm)
1	154.96
2, 6	127.22
3, 5	127.22
4	124.31
7	68.46
8	168.66
9	53.31
10	68.46
11	40.06
12	51.57
13	168.66
14	60.43
15	28.12
16	41.49
17	22.86
18	37.89
19	154.96
20	37.89
21	139.84
22, 26	127.22
23, 25	127.22
24	127.22
27, 28	16.76
29, 30	18.75



(a)



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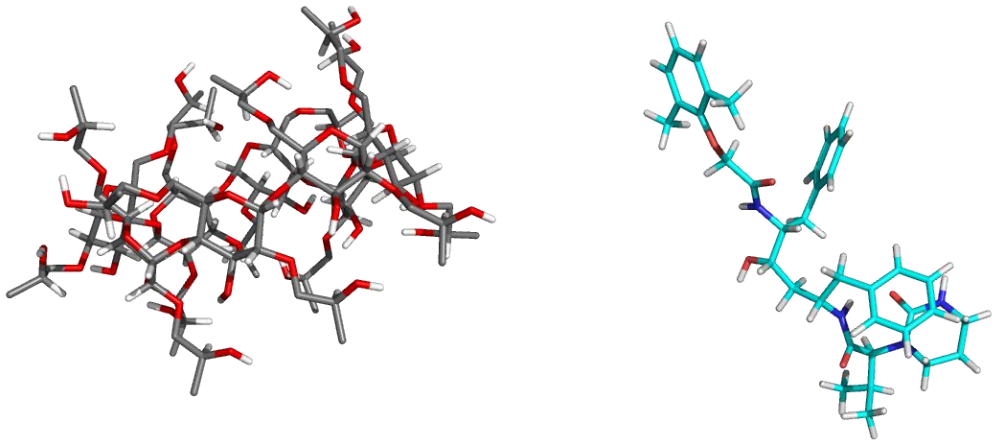

(c)

Figure. S5 Snapshots of the final CyD and LPV orientation after complexation (a) α-CyD, (b) HP-γ-CyD; and (c) HP16-γ-CyD

Table S7: Three-Dimensional form and size of evaluated CyD*

Properties	γ-CyD	HP-γ-CyD	HP*16*-γ-CyD
Internal diameter (Å)	12.6	9.8**	11.7
External diameter (Å)	13.8	10.2	11.45
Cavity height (Å)	6.6	11.1	13.5
Approx. cavity volume ($Å^3$)	819.3	832.6	1456.9

* **Notes to Table S7**

Fermeglia and co-workers have previously reported values of 11.8 Å and 5.7 Å for the internal diameter and cavity height of energy minimized β-CyD respectively [1]. While these values are similar to those presented here, they are significantly different from those commonly reported in literature [2].

** The reduction in the values of the internal and external diameters for both HP-γ-CyD; and HP16-γ-CyD is probably due to the loss of rigidifying effect of the circular network of intermolecular hydrogen bonds of γ-CyD after the substitution of its secondary faces with (2-hydroxyl)propyl. This derivatization induced conformational mobility and disruption of cavity sizes have been previously reported in literature [3,4]

References

1 Fermeglia, M. et al. (2003) Host-guest inclusion complexes between anticancer drugs and beta-cyclodextrin: Computational studies. *Carbohydrate Polymers* 53 (1), 15-44

2 Salustio, P.J. et al. (2011) Advanced Technologies for Oral Controlled Release: Cyclodextrins for Oral Controlled Release. *Aaps Pharmscitech* 12 (4), 1276-1292

3 Ellwood, P. et al. (1992) Conformational Mobility in Chemically-Modified Cyclodextrins. *Journal of Inclusion Phenomena and Molecular Recognition in Chemistry* 12 (1-4), 121-150

4 Yong, C.W. et al. (2008) Structural behaviour of 2-hydroxypropyl-beta-cyclodextrin in water: Molecular dynamics simulation studies. *Pharmaceutical Research* 25 (5), 1092-1099

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CHAPTER 4

Polymer cyclodextrin sub-microcarrier for oral drug delivery and enhanced antiretroviral activity of lopinavir

This chapter is based on a manuscript submitted for publication:

Oluwatomide Adeoye, Inês Bártolo, Jaime Conceição, Andreia Bento da Silva, Noélia Duarte, Ana Paula Francisco, Nuno Taveira and Helena Cabral Marques *Polymer cyclodextrin sub-microcarrier for oral drug delivery and enhanced antiretroviral activity of lopinavir*

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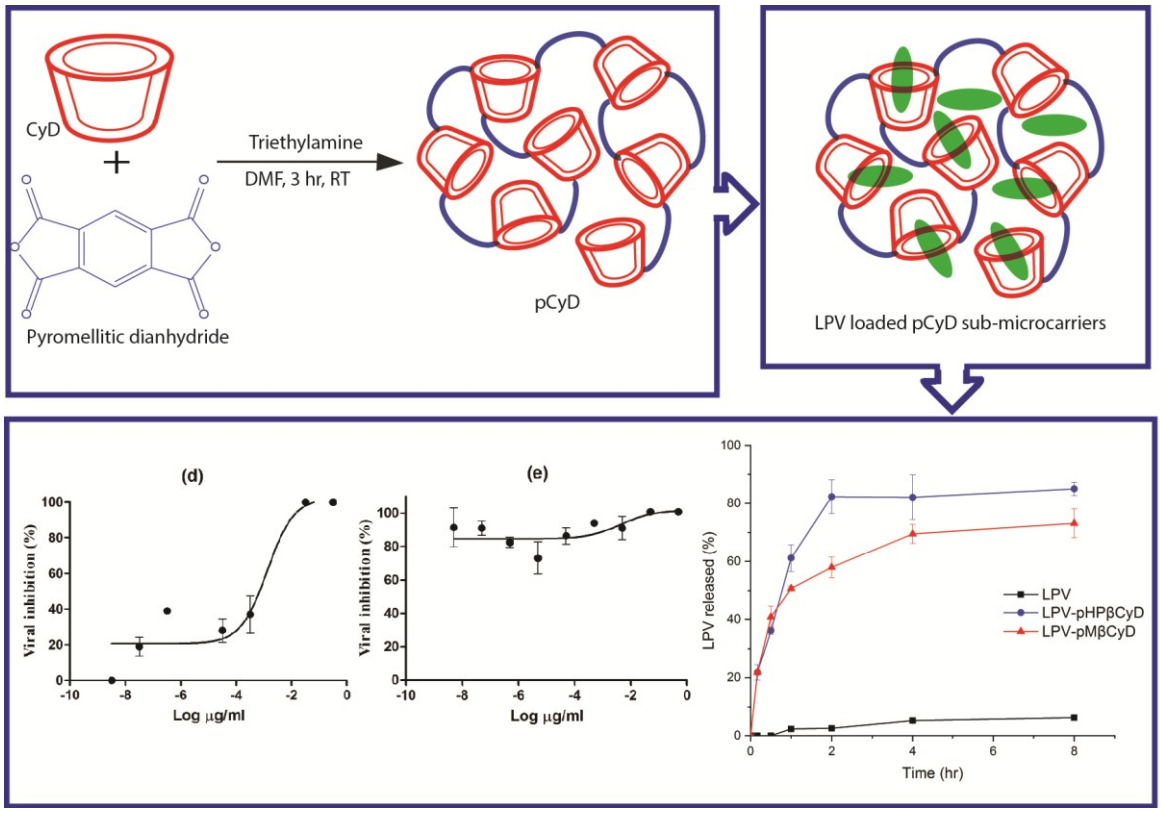
Abstract

Herein, we report the synthesis of a hyper-crosslinked cyclodextrin-polymer (pCyD) sub-microcarrier for the development of an oral lopinavir (LPV) delivery system. By using pyromellitic dianhydride (PMDA) as cross-linker and either (2-hydroxyl)propyl-β-, or methyl-β-cyclodextrin as monomers; two types of pCyD (pMβCyD, pHPβCyD) were synthesised and thoroughly characterized. LPV loaded pCyD were characterised using DSC, FTIR, XRD, HPLC-MS/MS and evaluated for particle size, zeta potential, cell-cytotoxicity and antiretroviral activity. Physicochemical characterization confirmed the synthesis of pCyDs, the molecular inclusion of LPV in pCyDs while drug release showed a 12-14 fold increase in the solubility of LPV. Cytotoxicity assays indicated that both pCyD and LPV loaded pCyD were safe. *In vitro* antiviral studies revealed a concentration independent antiretroviral activity for both pMβCyD and pHPβCyD exhibited (~80 -90 %), while LPV loaded formulations revealed synergistic antiretroviral especially with pMβCyD. The pCyDs may be act as functional drug delivery carriers for enhanced drug antiretroviral activity.

Graphical Abstract



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1. Introduction

Cyclodextrins (CyD) are cyclic oligosaccharides made up of 6, 7 or 8 glucopyranose units linked by α(1-4) glycosidic bonds. Their hydrophilic exterior surface and less hydrophilic interior cavity allows them to undergo host-guest molecular interaction with a wide variety of active pharmaceutical ingredients (APIs). Thus, they are able to form inclusion and non-inclusion complexes in order to solubilise lipophilic drugs and enhance their bioavailability and bio-activity. Over the past three decades, CyD have been used in the pharmaceutical industry to ameliorate the physicochemical and physiological stability of APIs, and to target and/or control *in vivo* drug release and pharmacologic activity [1-5]. Despite their utility however, parent CyD and their derivatives have some limitations related to their inclusion capability (especially for high molecular weight APIs), toxicity profiles, and their ability to efficiently control drug delivery [6-8]. In order to resolve some of these limitations, the highly reactive and easily functionalizable primary and secondary hydroxyl groups of CyD have been targeted for the synthesis of CyD polymer systems (pCyD). Various types of polymers such as epichlorohydrin, polyamidoimine, polyacrylic esters, polymethacrylates, diisocyanates etc. have been used for the synthesis of pCyD [8-11].

Cyclodextrin nanosponges are a subset of pCyDs that are water insoluble hyper-cross-linked, highly porous, crystalline or amorphous non-aggregating systems with a 3-dimensional mesh-like structural arrangement [12-15] . The CyD cavity and the mesh-like network formed by the cross-linked CyD monomers acts as nanodomains for the inclusion of both hydrophilic and hydrophobic drugs [12, 16]. Typically, a simple polymerization reaction between β-CyD and bi-functional agents such as carbonyl compounds (e.g. diphenyl carbonate) and organic dianhydrides (e.g. pyromellitic dianhydride, PMDA) [14, 15, 17] can be used to prepare pCyDs that are biodegradable, non-toxicity, stable at high temperatures (> 300 ^{o}C) and insoluble in both water and organic solvents [9, 17]. The synthesis of water soluble varieties has been achieved by reacting β-CyD and PMDA beyond the critical conditions that allow the gelation phenomenon to occur [18].

Whereas, scientific advances in HIV/AIDS pharmacotherapy have significantly enhanced disease prognosis and health related quality of life [20, 21], there are still no clinically

available nanotherapies able to integrate the huge advantages of nanomedicine such as improved drug bioavailability, targeted biodistribution and patient compliance into HIV/AIDS therapy [22-25]. Due to the versatility of pCyDs in the development of nanosystems for various types/classes of diseases [15, 26-28], they have huge potentials for addressing some of the bioavailability and biodistribution problems associated with HIV/AIDS therapeutics.

Herein, we report the synthesis of a pCyD based sub-microcarriers for the development of an oral antiretroviral drug formulation. Lopinavir (LPV), a HIV-1 protease inhibitor whose oral bioavailability is limited by low aqueous solubility and poor intestinal permeability was used as a model drug. LPV is currently co-formulated with suboptimal doses of ritonavir (RTV) which inhibits its P-glycoprotein (P-gp) and cytochrome P450 (CYP450 3A) mediated pre-systemic and systemic metabolism [29, 30]. However, the side effects of RTV such as lipid elevation, glucose intolerance, gastrointestinal intolerance and perioral paraesthesia has necessitated the development of a RTV free LPV formulation [31-34]. Also, paediatric formulations of LPV (even with RTV) is fraught with problems such as the presence of high quantities of ethanol and propylene glycol in the commercially available oral solution [24, 35]. Thus, we synthesised water soluble pCyD using PMDA and either of MβCyD and HPβCyD. The choice of both MβCyD and HPβCyD was based on previously reported role in the modulation of physiological and cell membrane properties for enhanced bioavailability and therapeutic efficacy [36, 37]. Colloidal complexes of LPV were prepared with the pCyD and the particles fully characterized for morphology, physicochemical properties, cytotoxicity and *in vitro* antiviral activity.

2. Materials and Method

2.1. Materials

LPV (molecular weight (MW) 628.81 g/mol) was a kind gift from Mylan (India) while MβCyD and HPβCyD were gifts from Roquette (France) and Wacker Chemie AG (Germany) respectively. PMDA, triethyleneamine (Et_3N) and polyvinyl alcohol (PVA) were purchased from Sigma Aldrich (Germany); while dimethylformamide (DMF) and acetone was purchased from Valente e Ribeiro (Portugal). All compounds and reagents were used as received without further purification.

2.2. Methods

2.2.1. Synthesis of PMDA/CyD polymer

To prepare water soluble hyper-crosslinked PMDA and CyD based pCyDs, 0.22 mM of anhydrous MβCyD or HPβCyD was dissolved in 5 ml of DMF under magnetic stirring at room temperature. After obtaining a clear solution, 3.59 mM of TEA was added to the reaction. Then 0.88 mM of PMDA was added and vigorously stirred at room temperature for 3 hr. Particles of the pCyD (i.e. pMβCyD and pHPβCyD) were then precipitated in excess acetone. The particles were repeatedly washed with acetone to remove residual by-products, recovered by vacuum filtration and dried under vacuum for 24 hr. The dried pMβCyD and pHPβCyD were kept in a dessicator for further use. Formation of pCyD was confirmed by spectroscopic (^{13}C CP/MAS NMR and Raman) and thermal analysis (Differential Scanning Calorimetry - DSC).

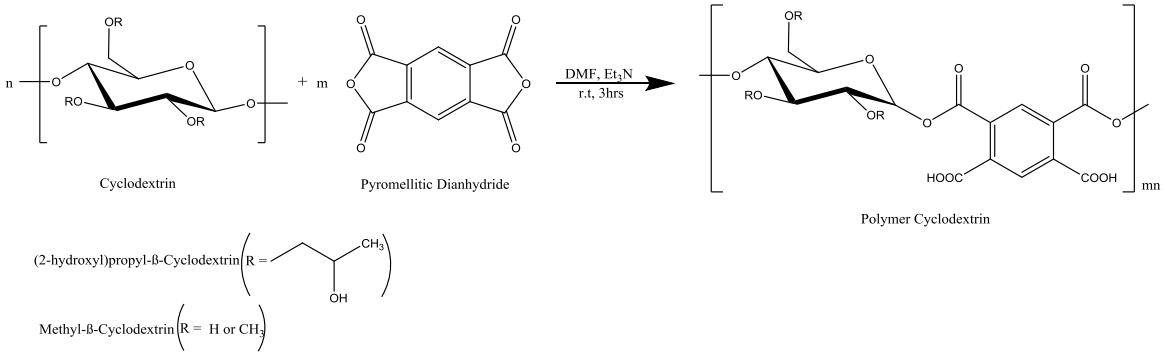


Fig. 4.1.1. Schematic presentation of pHPβCyD and pMβCyD synthesis.

2.2.2. Preparation of LPV loaded pCyD complexes

Colloidal complexes of pCyD and LPV were prepared by modifying a previously described nanoprecipitation-sonication method [38]. Briefly, 50 μL ethanolic solution of LPV (10 mg/ml) was added to 1 ml solution pCyD (5 mg/ml) in 2% PVA and then sonicated (BRanson digital sonifier UK). The organic solvent was removed and the LPV/pCyD colloidal suspensions were lyophilized to obtain dried particles.

2.2.3. Particle size distribution, zeta potential determination and surface morphology

The dynamic light scattering method was used to determine the hydrodynamic diameter (z-average) and polydispersity index (PdI) of LPV-pCyD complexes (Zetasizer® Nanoseries S, Malvern Instruments, UK) while the zeta potential was determined by electrophoretic light scattering analysis (Zetasizer® Nanoseries Z, Malvern Instruments, UK). All samples were suitably reconstituted and analysed at 25 °C. All measurements were performed in triplicates and results shown as mean ± standard deviation (SD).

The surface morphology of the pCyDs and LPV loaded pCyDs were examined by scanning electron microscopy (SEM). Images were acquired with a field emission microscope (JEOL, JSM-7001F, Japan) with an accelerating voltage of 15 kV. The samples were prepared by coating with gold using a coating system (Polaron, E5100, England).

2.2.4. Physicochemical characterization of LPV-pCyD complexes

Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectra of all samples were recorded in Perkin-Elmer Spectrum Two spectrometer (PerkinElmer, USA). Samples were placed on the diamond plate surface and scanned 16 times from 400 to 4000 cm^{-1} at room temperature.

The amorphization of the formulations was determined by X-ray diffraction (XRD) measurements on a Philips Analytical PW 3050/60 X'Pert PRO (theta/2theta) equipped with X'Celerator detector and with automatic data acquisition (X'Pert Data Collector (v2.0b) software), using monochromatized Cu-Kα radiation as incident beam, 40 kV–30 mA. The diffraction patterns were collected with a 2θ angle ranging between 5° and 40° and a scan rate of 0.02°/min.

2.2.5. Encapsulation Efficiency and *in vitro* drug release studies

To determine the LPV encapsulation efficiency of the formulations, lyophilized powders were dispersed in 50% ethanol and sonicated for 15 minutes to ensure complete dissolution. The solutions were then passed through a 0.45 μm membrane filter and the LPV content was determined by a previously reported HPLC-MS/MS method.

In vitro release of LPV from pCyDs were performed using dialysis compartment with a cross-sectional area of 0.81 cm^2. A dialysis membrane (Medicell dialysis tubing, cut-off

12–14 KDa, UK) was used for separation between the donor compartment and the recipient. The donor compartment was filled with a 1 mL dispersion of each sample and immersed in 10 mL phosphate buffer solution (PBS, pH 7.4). At predefined time intervals over a period of 8 hr, 1 ml aliquots were withdrawn and replaced with fresh phosphate buffer at the same temperature. LPV concentrations were assayed by HPLC-MS/MS.

2.2.6. Cell lines

Caco-2 (Human colon carcinoma), Sup-T1 (T cell lymphoblastic lymphoma) cells and TZM-bl (recombinant HeLa cell that expresses high levels of CD4, HIV-1 co-receptors (CCR5 & CXCR4) and integrated β-galactosidase and luciferase reporter genes under the control of HIV-1 long terminal repeat) cell lines were from American Type Culture Collection(ATCC, USA). The Caco-2 cells (American Type Culture Collection) were grown Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco/Invitrogen, USA), 1% (v/v) non-essential amino acids (Gibco/Invitrogen, USA) and 100 U/ml of penicillin-streptomycin (Gibco/Invitrogen, USA). TZM-bl cells (AIDS Research and Reference Reagent Program, National Institutes of Health, USA) and HEK293T cells (American Type Culture Collection) were cultured in complete growth medium that consists of Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco/Invitrogen, USA), 100 U/ml of penicillin-streptomycin (Gibco/Invitrogen, USA), 1mM of sodium pyruvate (Gibco/Invitrogen, USA), 2mM of L-glutamine (Gibco/Invitrogen, USA) and 1 mM of non-essential amino acids (Gibco/Invitrogen, USA). Sup-T1 cells (AIDS Research and Reference Reagent Program, National Institutes of Health, USA) were cultured in RPMI-1640 medium (Gibco/Invitrogen, USA) supplemented with 10% FBS (Gibco/Invitrogen, USA), 100 U/ml of penicillin-strepotmycin (Gibco/Invitrogen, USA),All cells were incubated at 37 °C in an atmosphere with 5% CO_2 and 95% relative humidity.

2.2.7. Cellular viability assays

The cytotoxic activity of free LPV, pCyD and LPV loaded pCyDs on Caco-2 and SupT1 cell lines was evaluated using the AlamarBlue cell viability reagent (Life Technologies, USA) [39]. Cells were individually seeded into a 96 well culture plate at a density of 1×10^5 cells/well and incubated for 24 hours. Then, the compounds were added at 2 different concentrations and incubated for 24 and 48 hours. At least, two independent

experiments were performed for each cytotoxicity analysis. For each assay, medium controls (only growth medium), cell controls (cells without test compounds) and cytotoxicity controls (sodium dodecyl sulphate) were used. Cell viability for each compound was performed in triplicate wells and expressed in average percentage of absorbance of treated cells compared with control.

2.2.8. Antiviral assays

The HIV-1 SG3.1 strain used in this study was obtained by transfection of HEK293T cells with pSG3.1 plasmid using jetPrime transfection reagent (Polyplus-tranfection SA, Illkirch, France) according to the manufacturer's instructions. The 50% tissue culture infectious dose ($TCID_{50}$) of the virus was determined in a single-round viral infectivity assay using a luciferase reporter gene assay in TZM-bl cells [47, 64] and calculated using the statistical method of Reed and Muench [65].

The antiviral activity of the compounds was determined in a multi-cycle viral infectivity assay using Sup-T1 cells and the subsequent infection of TZM-bl cells. Briefly, the SUP-T1 cells were seeded into a 96 well culture plate at a density of 2×10^4 cells/well in the presence of serial fold dilution of each compound and incubated for one hour. Then, the cells were infected with 200 $TCID_{50}$ of HIV-1 SG3.1 strain.. After 72 hours, the supernatant*s* were collected. and.. Then The antiviral activity of those supernatants was determined in a single-round viral infectivity assay using TZM-bl reporter cells, as previously described [47, 66]. After 48h of infection, luciferase expression was quantified with Pierce Firefly Luc One-Step Glow Assay Kit (ThermoFisher Scientific, Rockford, USA) according to the manufacturer's instructions. At least two independent experiments were performed for each antiviral activity analysis. For each virus and compound dilution, the assay was set up in triplicate wells. The 50% inhibitory concentration (IC_{50}) was estimated from the sigmoidal dose-response (variable slope) equation in Prism version 4.0c for Macintosh (GrahPad Software, San Diego, CA, USA).

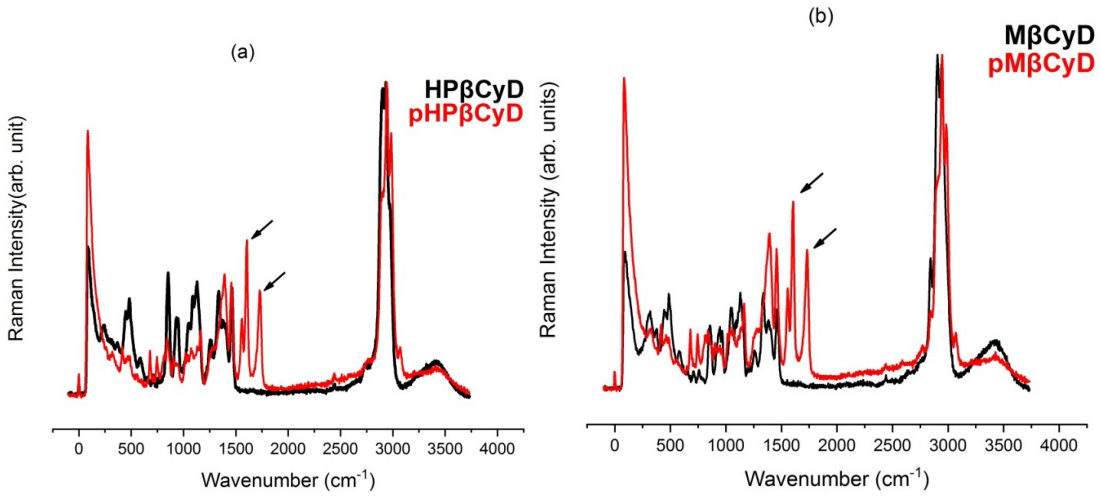
3. Results and Discussion

3.1. Structural and thermal characterization of pCyDs

The nucleophilic attack of the hydroxyl groups of CyD monomers during the condensation polymerization reaction is the main mechanism formation of pCyD [41]. Both ^{13}C NMR and Raman spectroscopy were used to structurally confirm the successful cross-linking of the CyD monomers while DSC was used to evaluate changes in thermal properties of the new polymers. The spectra obtained from the ^{13}C CP/MAS analysis of the synthesised pMβCyD and pHPβCyD were assigned as previously described in literature [42-44].

Table 4.1.1: ^{13}C Chemical Shift of pCyD

Peaks	^{13}C Chemical Shift			
	HPβCyD	pHPβCyD	MβCyD	pMβCyD
C 1	102.66	102.52	102.99	102.70
C 2, C 3, C 5	73.55	73.59	73.58	73.48
C 4	82.30	82.34	83.43	83.45
C 6	61.59	60.94	61.34	61.19
R1	67.54	67.30		
R2	20.11	20.22		
CBL (Carbonyl)	-	171.35	-	172.34
	-	47.38	-	47.38



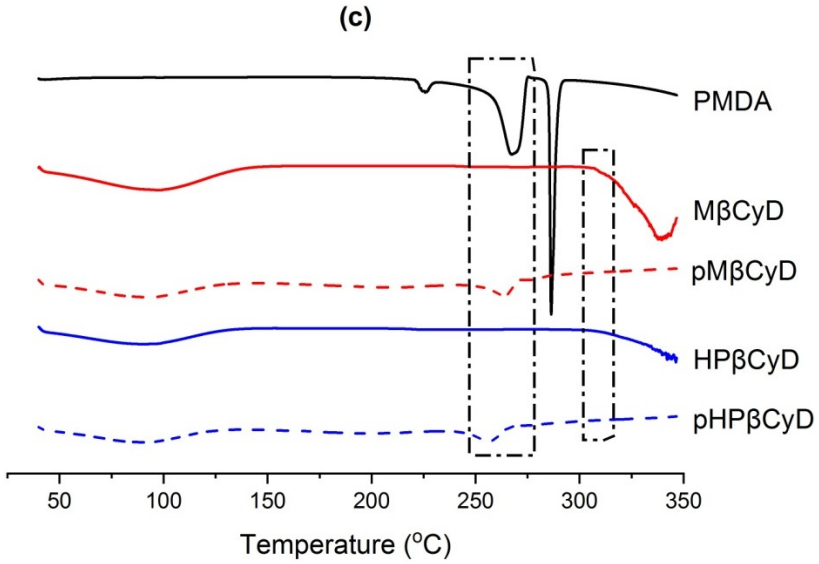


Fig. 4.1.2. (a, b) Raman spectra of HPβCyD, pHPβCyD, MβCyD and pMβCyD, (c) DSC thermograms of PMDA, MβCyD, pMβCyD and HPβCyD, pHPβCyD

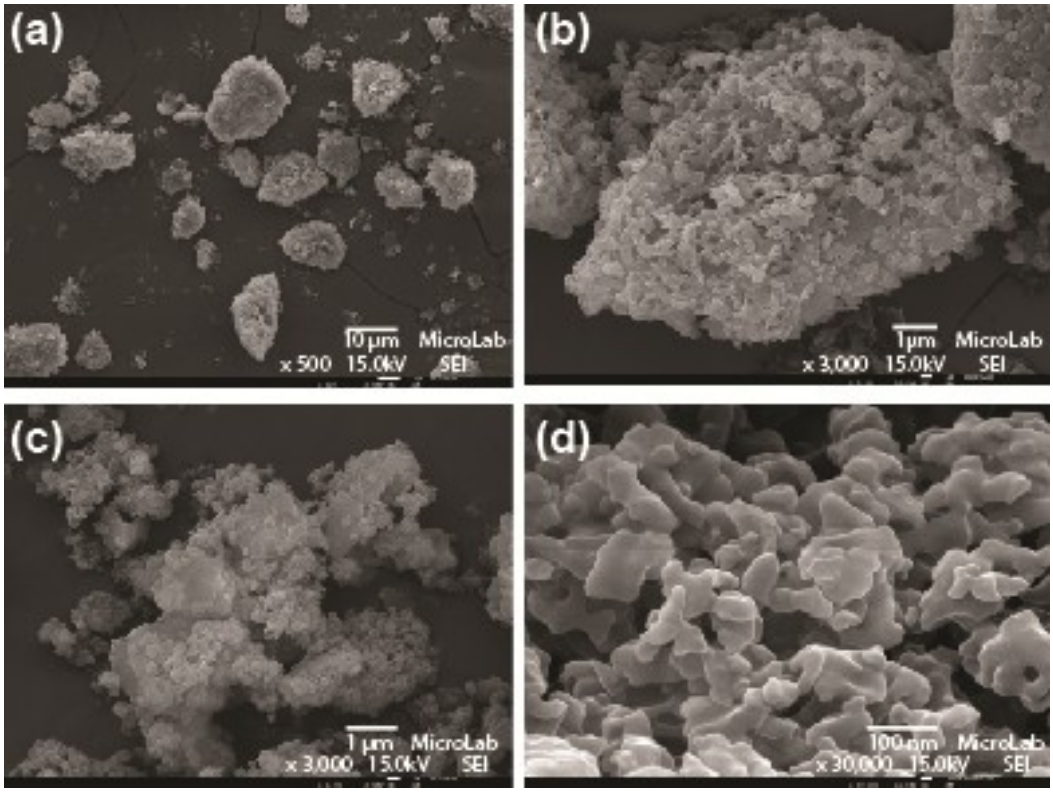
The appearance of the carbonyl groups (C=O) of PMDA around 170 ppm was confirmatory for the formation of pCyD as shown in Table 1. No significant deviations on chemical shift was observed in Carbons C 1, C4, C 2, C 3, C 5, suggesting that the sugar rings of parent CyDs retained their local environment and conformation in the synthesized polymers [44, 45]. Also, the presence of additional peaks below 50 ppm has been reported as confirmatory for the successful linkage of primary hydroxyl groups of CyD after pCyD synthesis [46]. Raman spectroscopy is an ideal tool for the analysis pCyD formation as it presents well resolved vibrational modes for functional groups introduced by the crosslinker [47]. As shown in Fig. 4.1.2(a, b), the Raman spectra reveals the clearly separated stretching dynamics of both the unsaturated PMDA ring (ca.1550-1610 cm^{-1}) and those of its carbonyl groups (ca.1725-1735 cm^{-1}) [47, 48]. Thermal analysis (Fig. 4.1.2c) of the synthesised pCyD revealed a different thermal transition compared to those of starting CyDs materials. While the thermogram of MβCyD and HPβCyD showed an endothermic peak around 300 °C representing their decomposition as previously reported in literature [49, 50], those of pMβCyD and pHPβCyD increased beyond 300 °C suggesting a change in the thermal stability of synthesised pCyD. Also, additional melting endothermic peaks were added to the pCyD between 250 °C and 280 °C signifying the integration of PMDA into the new polymer.

3.2. Characterization of LPV loaded pCyD complexes

The antiretroviral drug LPV is considered a poor candidate for drug development due the aforementioned problems [51, 52]. The challenging nature of LPV formulation is best underlined by the fact that its paediatric formulations requires 42.5% of ethanol and 15% propylene glycol to maintain LPV solubility in formulation [52, 53]. Typically, drug loading into PMDA-CyD based pCyD delivery systems is done by shaking a dispersion of the drug in pCyD for a approximate time (depending on the drug) and subsequently lyophilization the filtered aliquot to obtain dry powder [14].

Table 4.1.2: Mean particle size, polydispersity index, zeta potential and entrapment efficiency of LPV loaded pCyDs

LPV Formulations	Particle size (z-average nm)	Polydispersity index (PdI)	Zeta potential (mV)	Encapsulation Efficiency (%)
pHPβCyD	515.30 ± 11.11	0.235 ± 0.016	-10.05 ± 0.73	85.03 ± 0.83
pMβCyD	560.53 ± 11.66	0.199 ± 0.075	-9.41 ± 0.08	54.37 ± 4.35



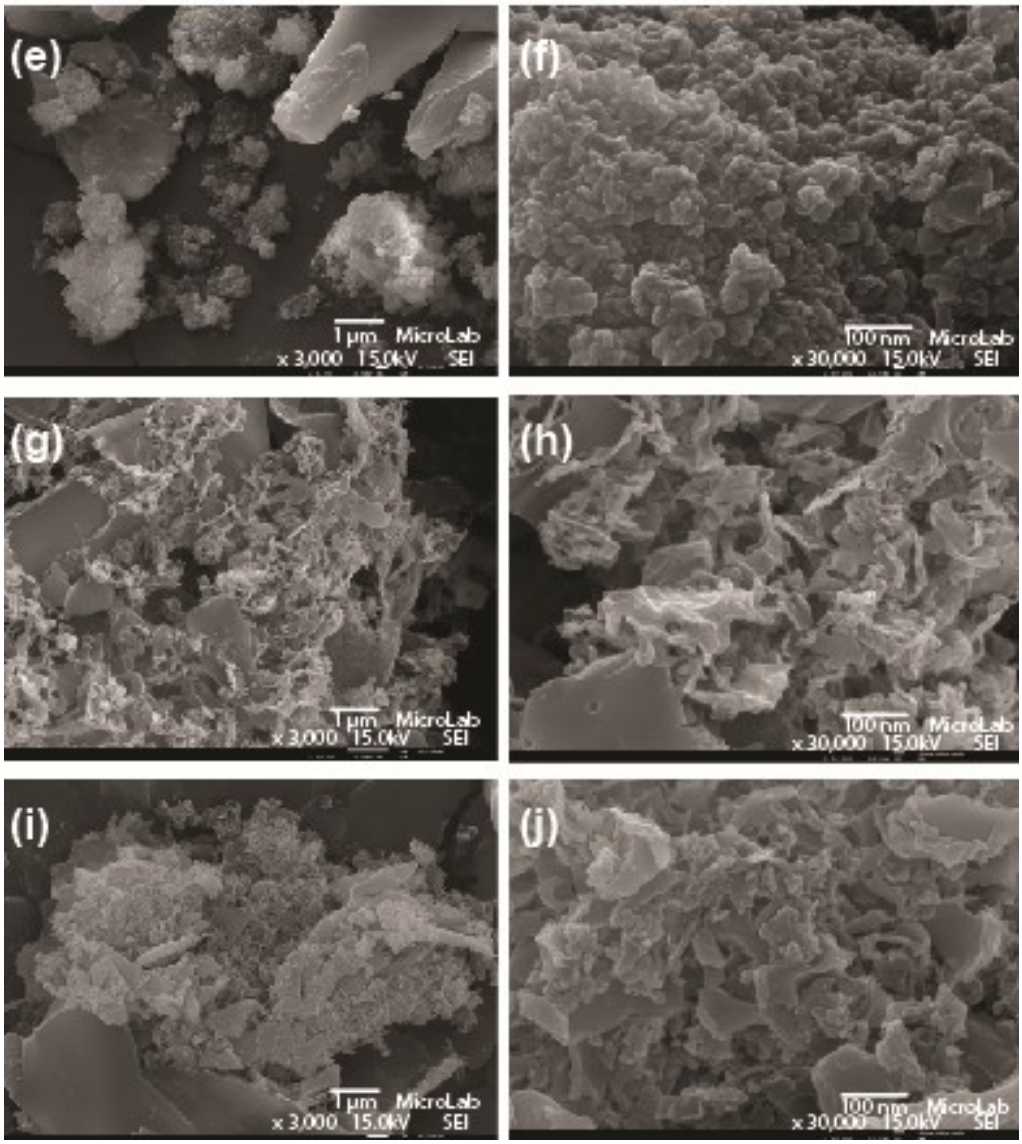


Fig. 4.1.3. SEM micrographs of: (a, b) LPV, (c, d) pHPβCyD, (e, f) pMβCyD (g, h) LPV-pHPβCyD, and (i, j) LPV-pMβCyD; at different magnifications

Because LPV is practically insoluble in water, this approach resulted in < 10% drug LPV encapsulation. Thus, a sonication assisted precipitation method was employed for the preparation of LPV-pCyD complexes, whereby ~5% ethanol and a 2% PVA solution were used as the LPV solvent and non-solvent stabiliser of the colloidal system respectively. As shown in Table 2, the particle sizes of pCyD formulations after reconstitution are in sub-micron range with a narrow size distribution. Drug encapsulation efficiency of the LPV loaded pMβCyD and pHPβCyD and the zeta potentials are presented Table 2. Also, the surface morphology of the pure LPV, plain pCyDs and LPV loaded pCyDs examined by SEM (Fig. 4.1.3(a-j) reveals the highly porous and rough structures of the pCyDs. This rough and porous surface morphology was retained in the

drug loaded samples (g-j) suggesting its ability to reduce LPV crystallinity and increase its solubility.

Figure 4.1.4a shows the ATR-FTIR spectra of the pure LPV, plain pCyDs and LPV loaded pCyDs. The spectra of pCyDs displays the characteristic stretching vibrations of PMDA's anhydride C=O and aromatic C=C group at ~1727 cm^{-1} and ~1590 cm^{-1} respectively, while the characteristic C=O stretching vibration of LPV's amide group was observed at ~1610-1662 cm^{-1} [41, 54].

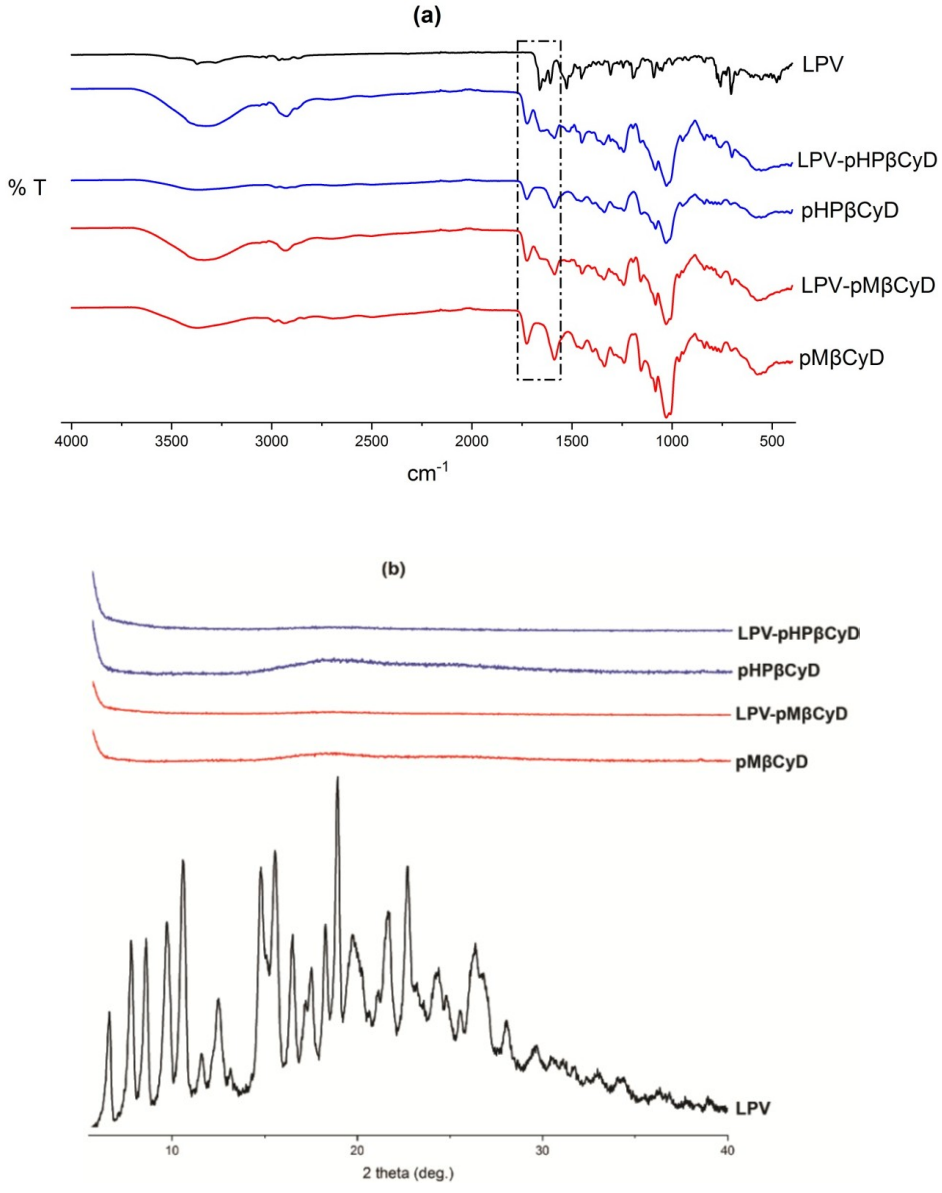


Fig. 4.1.4 (a) AT-FTIR spectra of LPV, pHPβCyD, pMβCyD and their LPV loaded complexes; and (b) X-ray diffractograms of LPV, pHPβCyD, pMβCyD, PVA and their LPV loaded complexes

The broadening of reduced intensity of LPV's C=O stretching vibrations in the spectra of the drug loaded pHPβCyD and pMβCyD suggests some form of molecular interaction between the pCyD and LPV. As shown in Figure 4.1.4b, the XRD diffraction patterns of LPV reveals sharp and intense peaks indicating the crystalline structure of the drug, while those of the pHPβCyD and pMβCyD indicates their complete amorphous nature. After drug loading, the XRD patterns of drug loaded pHPβCyD and pMβCyD revealed a complete loss of LPV crystallinity suggesting the complete amorphization of LPV and a consequent increase in its solubility.

3.3. *In vitro* release of LPV from pCyD

The *in vitro* drug release profile of pure LPV and the LPV loaded pCyDs are presented in Figure 4.1.5. Both pMβCyD and pHPβCyD facilitated ~12 and ~14 fold increase in the dissolution of LPV. The complete loss of LPV crystallinity observed XRD study and the sub-micron particle sizes of the formulations may be responsible for the significant increase in LPV solubility.

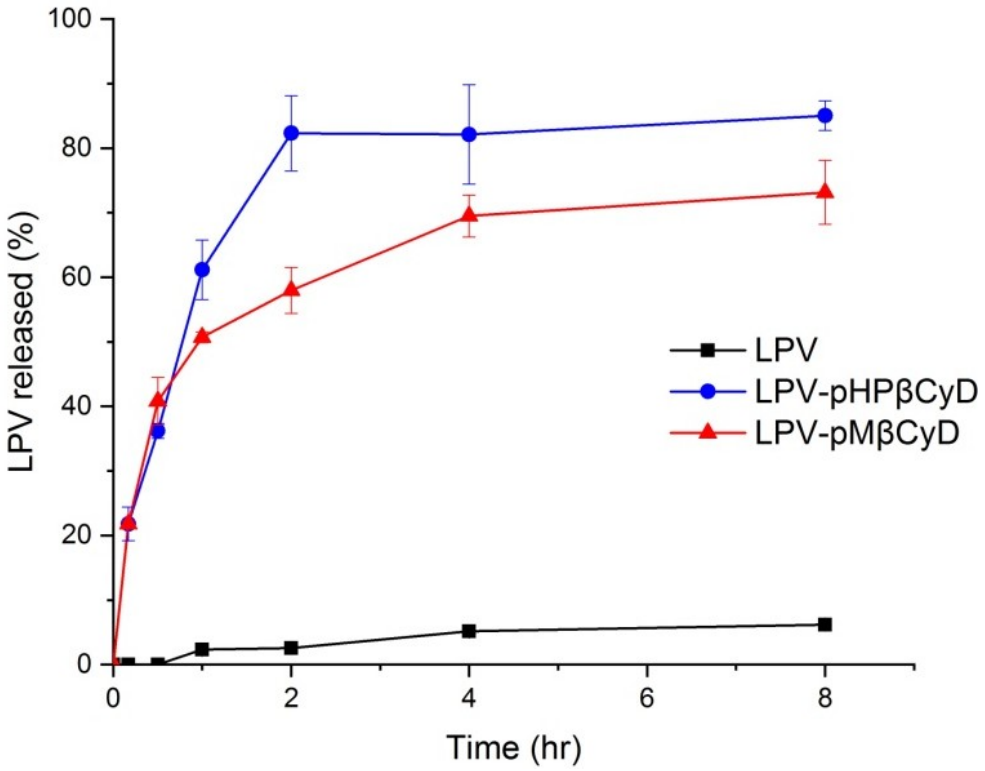


Fig. 4.1.5 Release profile of LPV from pCyD.

3.4. *In vitro* cytotoxicity and antiretroviral activity

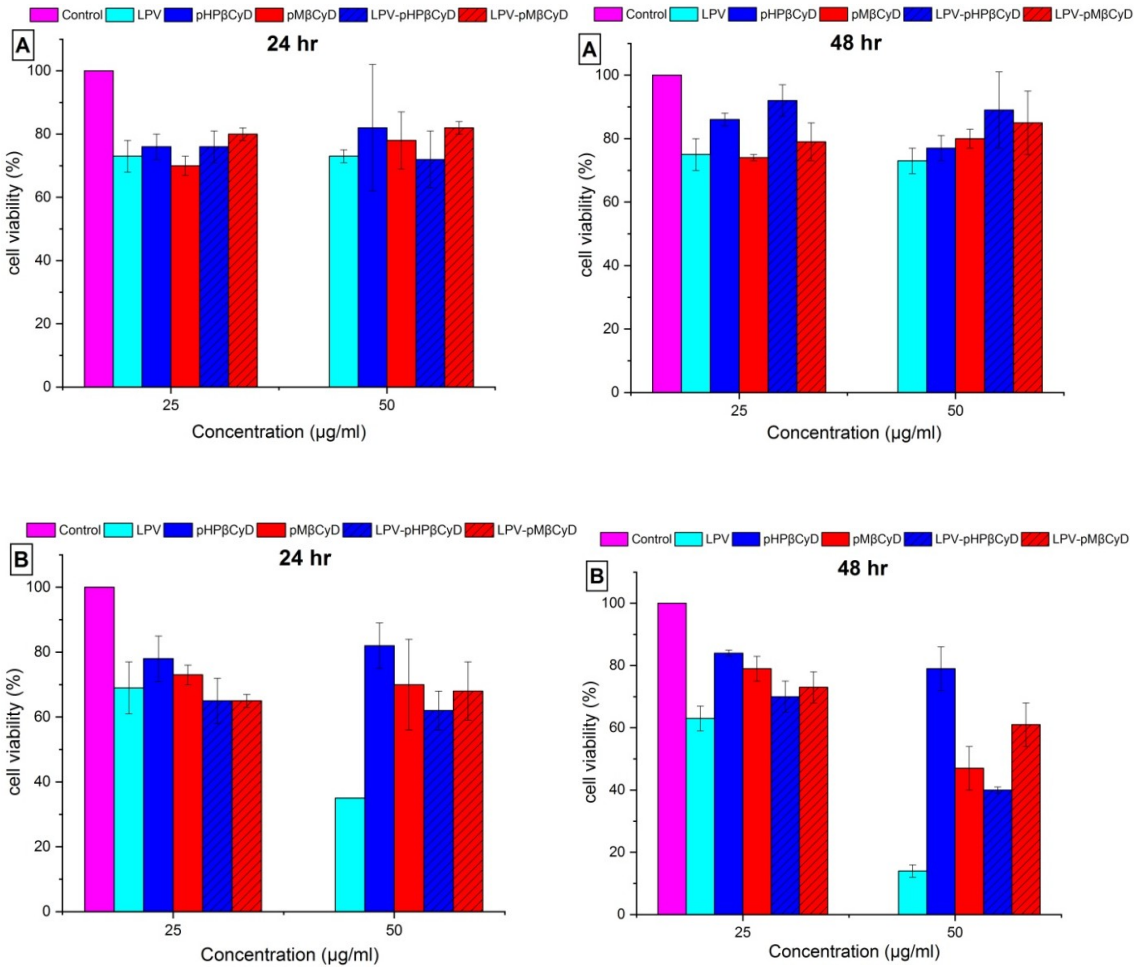


Fig. 4.1.6 Cell viability graphs of LPV, pHPβCyD, pMβCyD and their LPV loaded complexes in (A) Caco-2, and (B) SUP-T1 cell lines; after 24- and 48 hr incubation at 25- and 50 µg/ml.

The survival profiles of Caco-2 and Sup-T1 cells after 24- and 48 hr exposure to the pure LPV, pCyDs and LPV loaded pCyDs are presented in Figure 4.1.6. Several studies have reported the safety of pCyD prepared with β-CyD on cell cultures[55, 56]. However, due to MβCyD and HPβCyD's superior ability to interact with cell membrane lipids thus producing a concentration dependent cytotoxic effect [57, 58], they have been found useful as gastrointestinal permeation enhancers in oral drug development [36]. Our results suggest that the crosslinking of several monomers of these CyDs in the preparation of pCyDs did not increase their cytotoxicity compared to non-crosslinked MβCyD and HPβCyD [57] thus allowing their use as previously described[36]. In Sup-T1 cells, while the cytotoxic effect of LPV were concentration and time dependent, pCyDs produced a

similar toxicity profile to those observed in Caco-2 cells. The improvement in cell viability observed after the exposure of Sup-T1 to drug loaded pCyD may be related to a lower amount of free drug compound present in the formulation.

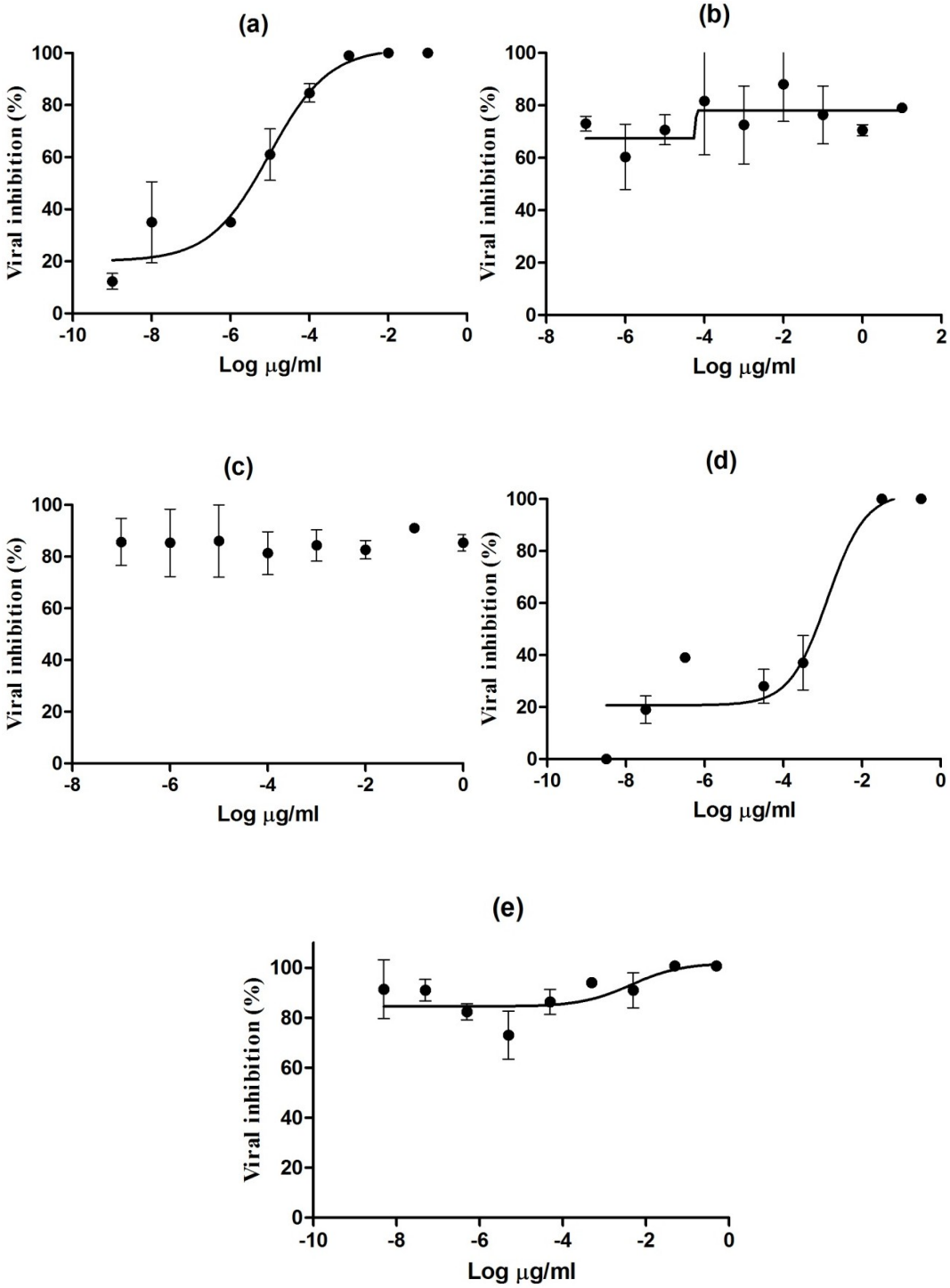


Fig. 4.1.7 Antiretroviral activity and dose response curve obtained for (a) LPV, (b) pHPβCyD, (c) pMβCyD, (d) LPV-pHPβCyD, and (e) LPV-pMβCyD

The ability of the pHPβCyD and pMβCyD and their LPV formulations to inhibit HIV-1 replication was evaluated by propagating the viruses in SUP-T1 cells and determining the viral infectivity of the supernatants in TZM-bl reporter cells. As shown in Figure 4.1.7(a,b,c), while pure LPV produced a typical sigmoidal dose response curve, both pHPβCyD and pMβCyD showed a dose independent HIV-1 inhibition (~80 -90 %). This viral inhibition by pHPβCyD and pMβCyD is consistent with previous reports where the membrane lipid sequestering ability of CyDs was used to demonstrate the importance of virion associated- and host membrane cholesterol in the fusion and infectivity of HIV-1 [59, 60]. After drug loading with LPV, the results suggests a change in the mechanism of viral inhibition. For LPV-pHPβCyD formulation (Figure 4.1.7d), the typical dose-response curve suggests that the presence of LPV in the cavities of pHPβCyD reduces the polymer's ability to sequester cholesterol. For LPV-pMβCyD formulation (Figure 4.1.7e), the result suggest that cholesterol sequestering was the main mechanism for viral inhibition and that synergistic effect with LPV allowed for 100 % viral inhibition.

4. Conclusions

This study demonstrated the possibility of synthesising water soluble hyper-crosslinked pCyDs using HPβCyD and MβCyD monomers, and PMDA crosslinker; and the application of the pCyD for the development of oral LPV drug delivery system. The physicochemical analysis of the synthesised polymers revealed the successful crosslinking of the CyD monomers by PMDA, and the complete amorphization of LPV in drug-pCyD formulations. Poly-dispersed sub-micron particles sizes, good encapsulation efficiencies and a significant increase in LPV release were observed in prepared formulations. The synthesised pCyDs represents a exhibited a concentration independent antiretroviral activity against HIV-1. The LPV loaded pMβCyD exhibited a synergistic antiviral activity thus indicating the promising. Considering the potential for drug dose reduction without a compromise on antiretroviral activity, these pCyDs represent a promising approach for developing good alternatives to clinically available antiretroviral drugs.

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CHAPTER 5

Concluding remarks and future work

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1. Concluding remarks

The work described in this dissertation explored the utility of cyclodextrin (CyD) for the development of oral antiretroviral drug delivery systems using lopinavir (LPV) as the model drug. Firstly, a computational approach was developed, validated (using ibuprofen as model drug) and then used to study the molecular interaction between LPV and several CyD molecules in order to predict the best CyD for developing a LPV drug delivery system. Secondly, supercritical fluid assisted spray drying (SASD) and the potential for using supercritical CO_2 to achieve ternary complex formation was explored as a method of choice for the preparation of drug-CyD complexes. Thirdly, water soluble hyper-crosslinked cyclodextrin-polymer (pCyD) sub-microcarriers were synthesized and evaluated for their ability to enhance LPV bioavailability and antiviral activity.

The preliminary studies on the computational approach and SASD processing technique described in chapter 2 and the application of the strategy espoused therein for the preparation of LPV-CyD complexes (Chapter 3), revealed the predictive utility of computational methodologies for CyD selection in the development of CyD based drug delivery systems. Using molecular docking and dynamics, it was possible to evaluate several factors implicated in CyD host guest molecular interaction with LPV and to select the best CyD for preparing LPV complexes. It was also possible to completely avoid the tedious and time/resource consuming traditional approach of selecting CyD by calculating association constants from phase solubility studies or isothermal titration calorimetry. Considering that the traditional approach for CyD selection in drug development relies heavily on the personal experience of the pharmaceutical scientist and a series of trial and error experiments for screening drug-CyD interaction; the successful identification and synthesis of the commercially unavailable derivative of HP-γ-CyD (i.e. HP17- γ-CyD) suggests that the application of *in silico* methodologies is a feasible approach for the rational and/or deductive development of CyD drug delivery systems. It also emphasises the potential for a drug specific *in silico* exploration of CyD host-guest molecular interactions beyond the limits set by the commercially availability of CyD derivatives (i.e. their isomers or DS patterns). While the immediate limitation of this approach may be the requirement for regulatory approval of new CyD derivatives, it has the potential for optimizing drug formulation especially when such drug-CyD complexes are part of hybrid drug delivery systems. Additionally, the approach employed herein has enormous industrial potential since it can allow for a shift in objectives of pre-

formulation scientists from a detailed thermodynamic analysis of CyD complexes to cost/time effective pre-identified/less-tedious endpoint physicochemical properties such as drug solubilization and amorphization. With regards to the SASD studies in Chapters 2 and 3, the observed ability of this clean, thermolabile API friendly and pharmaceutically scalable processing technique to enhance drug amorphization and solubilization (relative to CoEva) reveals the ternary complexation effect of supercritical CO_2 in enhancing CyD complexation of pharmaceuticals. An important limitation of the results obtained here relates to high CyD formulation bulk if our complexes are to be used for developing solid dosage forms. This will not be an important consideration when developing liquid dosage forms for paediatric use.

The development of LPV loaded pCyD colloidal complexes is described in Chapter 4. Our results demonstrated the possibility of synthesising water soluble hyper-crosslinked pCyDs using HPβCyD and MβCyD as monomers, and PMDA crosslinker. The synthesized polymers facilitated the complete amorphization of LPV in the LPV-pCyD formulations and a 12 and 14 fold increase (for pMβCyD and pHPβCyD, respectively) in dissolution of LPV. Since the choice of both HPβCyD and MβCyD as monomers for constructing pCyD sub-microcarriers was based on their ability to modulate physiological and cell membrane properties, an appropriate balance between potential cytotoxicity and desired activity is required. The cytotoxicity assays revealed the crosslinking of several monomers of the starting CyD molecules did not increased cytotoxicity. The observed dose independent antiviral activity of pMβCyD and pHPβCyD and the synergistic antiviral effect shown by pMβCyD formulations (which largely maintained dose independent viral inhibition effect) shows the feasibility of utilizing these polymers for antiviral drug dose reduction. A significant limitation to the results discussed in Chapter 4 related to ability of pCyD to mediate gastric permeability of LPV without the need for ritonavir (RTV). If we are able to show a substantially increase LPV bioavailability without sub-optimal doses of RTV, the synthesized polymers may have enormous potential for further development of new LPV delivery systems.

2. Future work

The study described in Chapter 4 is limited by the data on the ability of the synthesized pCyD to modulate gastric permeability. Thus, first set of supplementary work will be

focussed on evaluating the pCyD mediated gastric permeability of LPV using *in vitro* Caco-2 cell and *ex vivo* models. As previously mentioned, the identified problems of paediatric LPV formulations creates an opportunity to develop liquid dosage forms (solutions or suspensions) using the newly synthesized HP17-γ-CyD derivative or pCyDs.

Another important offshoot of the present study is the application of pCyD to the development of anti-HIV microbicides. β-CyD based pCyD (also synthesized but not used for this study due to our inability to control particle size after drug loading) are able to form gels. However, their ability to facilitate a synergistic and/or repeated viral inhibition would have to be established. Alternatively, antiviral drug loaded pMβCyD or pHPβCyD may be incorporated into other gel forming pharmaceutical excipients for further development.

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